pH and vascular smooth muscle tone

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pH And Vascular Smooth Muscle Tone

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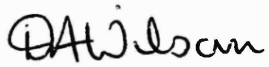

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And finally, to my family whose support has come in many guises and to Julie for keeping me sane over the past year or so.

Some people think that football is a matter of life and death....

I can assure them it is much more serious than that.

Bill Shankly

CONTENTS

SUMMARY

Acidosis is a condition that has been demonstrated to occur during myocardial ischaemia. This study was performed to further investigate the effects acidosis on the coronary circulation of the isolated perfused rat heart. Experiments were also performed on other vascular preparations (coronary and non-coronary) to compare the effects of acidosis.

Using the isolated perfused rat superior mesenteric bed, it was demonstrated that the responses to acidosis were dependent on the constrictor agent used.. Different effects were observed when intracellular pH and both intracellular and extracellular pH were reduced.

Decreases in intracellular pH in isolated rat hearts caused different effects on CPP. Withdrawal of NH_4Cl (leading to intracellular acidosis) caused a reduction in DT and an increase in CPP. In contrast, intracellular acidosis induced by butyrate application caused a decrease in DT and CPP.

In both isolated guinea pig and rat hearts, acidosis (whether metabolic, respiratory or using HEPES-buffered Tyrode) reversibly decreased DT in a pH-dependent manner (pH 7.4-6.2). In guinea pig hearts acidosis produced a sustained decrease in CPP. In contrast, acidosis produced a sustained constriction in the rat heart. Further species difference were revealed when K^+ constricted porcine coronary artery rings relaxed in response to metabolic acidosis.

In the rat heart the sustained constriction was insensitive to the adenosine antagonist 8-phenyltheophylline (10μM), the α-adrenoceptor antagonists phentolamine (10μM), the cyclo-oxygenase inhibitor indomethacin (10μM) and NO synthase inhibitor N0-ARG (100μM. It was found that the sustained acidosis-induced coronary constriction in the rat heart was sensitive to the removal of extracellular Ca^{2+} and the application of L-type Ca^{2+} channel blockers. Control experiments using hypoxia and the isopropyl ester of palmitoyl carnitine (P1Pi) to dilate the coronary would suggest that the inhibitory effect of nifedipine was due to Ca^{2+} channel block. Furthermore, data obtained with the Ca^{2+} channel agonist BAYK 8644, and endothelin-1 also suggests that acidosis activated L-type Ca^{2+} channels to cause Ca^{2+} influx and contraction of the vascular smooth muscle.

It was found that there is heterogeneity between coronary circulations of guinea pig, porcine and rat coronary circulations with respect to their responses to pH changes. It is proposed that in the rat heart acidosis increases CPP by blocking one or more K^+ channels and this opens L-type Ca^{2+} channels to cause coronary constriction.

INTRODUCTION

1.1 pH Regulation

pH regulation is vital to maintain cellular homeostasis and hence the performance of the cell. If a resting membrane potential of -60 mV is assumed, and extracellular pH is 7.4, intracellular pH would be in the region of 6.0-6.5, considerably more acidic than the resting figure of approximately 7.1-7.2 (Wray 1988). This implies that there must be one or more proton extrusion mechanisms working against the proton electrochemical gradient.

Acids, or their equivalents, are constantly being produced by the body through a number of metabolic pathways. In the long term, the body must rid itself of these potentially harmful by-products. One of the major pathways for proton elimination is via buffering with HCO_3^- and the conversion to CO_2 and H_2O, both of which are removed via the lung. This, plus renal control of hydrogen ion concentrations contributes to the overall control of the plasma pH. Plasma proteins and haemoglobin are also involved in the overall maintenance of pH, along with intracellular proteins. There are further cellular mechanisms responsible for maintenance of cellular pH. Efficient and adaptable intracellular pH regulation mechanisms are imperative for both cardiac muscle and vascular smooth muscle to maintain their function under times of metabolic stress *e.g.* during myocardial ischaemia. In order to achieve this, intracellular pH is usually maintained by various different mechanisms acting in concert (see Figure I1). The relative contributions of these various mechanisms differ depending on conditions including the cellular environment. These will be discussed below.

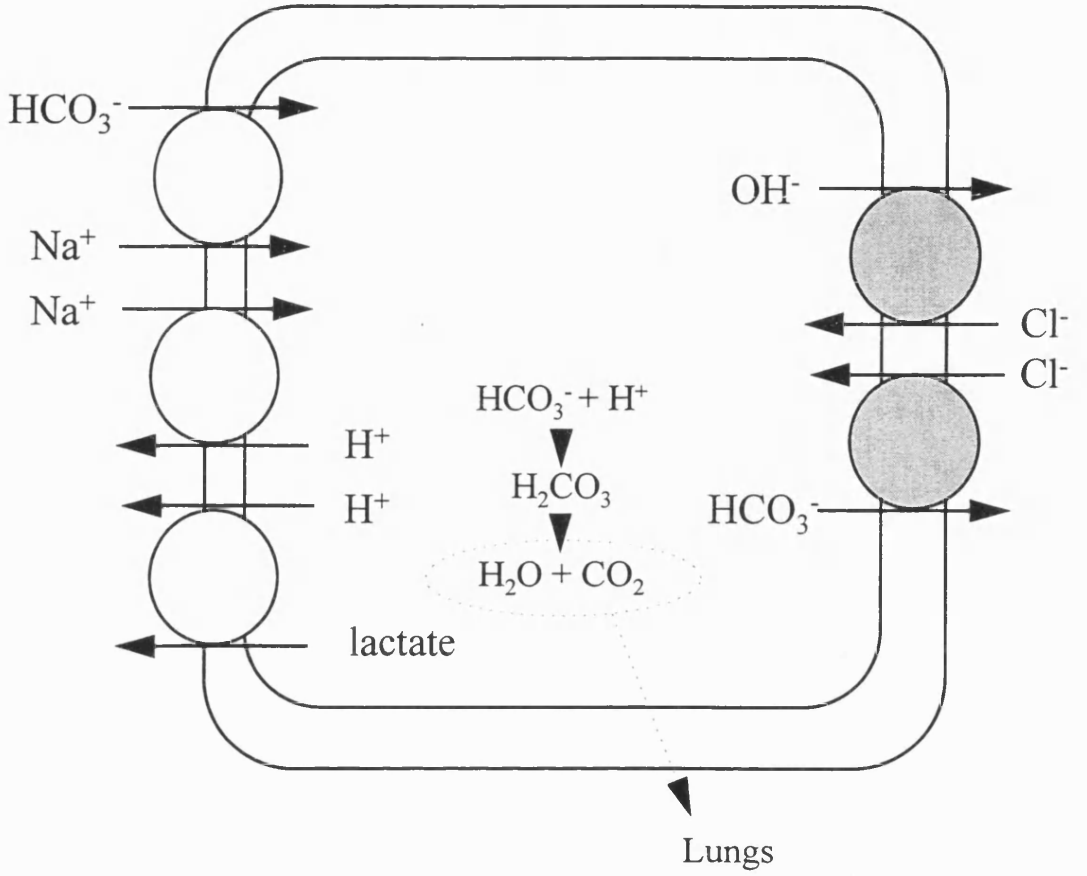


Figure I1: Diagrammatic representation of mechanisms responsible for maintenance of pH under resting conditions in cardiac cells. Open circles represent acid efflux mechanisms and shaded circles represent acid influx mechanisms.

1.1.1 Cardiac Muscle

Experiments performed with cardiac myocytes from a variety of species under various experimental conditions have demonstrated multiple pathways of intracellular pH regulation. One common method used to study pH regulation is the NH_4Cl wash-out technique. This causes intracellular acidosis whilst maintaining extracellular pH (see figure I2). In cultured rat ventricular myocytes, recovery of intracellular pH from acidosis induced by the wash-out of NH_4Cl-prepulse was completely inhibited by removal of extracellular sodium, and amiloride, a Na^+-H^+ antiport inhibitor (Weissberg *et al.*, 1989).

Additional pathways to the Na^+-H^+ antiport were confirmed in a similar study using rabbit ventricular myocytes (Nakanishi *et al.*, 1992). In this, recovery from NH_4Cl wash-out was completely inhibited by ethyl-isopropyl amiloride (EIPA). However, in HCO_3^-/CO_2 buffers, an additional 4-acetamido-4'-isothiocyanatostilbene-2' ,2' -disulfonic acid (SITS)-sensitive exchanger, the Na^+-independent $Cl^-HCO_3^-$ exchanger was revealed. A further study using another stilbene acid derivative, 4,4' -diisothiocyanatostilbene -2 ,2' -disulfonic acid (DIDS) in isolated rat ventricular myocytes showed that a Na^+-HCO_3^- co-transporter was also involved in intracellular pH regulation (Wu *et al.*, 1994). However, the rationale of using HCO_3^--free buffers to inhibit HCO_3^--dependent systems was questioned in this study as it was demonstrated that cellular CO_2 production can activate these HCO_3^--dependent systems. Similar EIPA and SITS-sensitive mechanisms have been demonstrated using intact perfused ferret hearts (Kusoska *et al.*, 1994; Grace *et al.* 1993). In addition, lactate and CO_2 washout

have been proposed as further pH recovery mechanisms following global ischaemia in ferret hearts (Vandenberg *et al.,* 1993).

1.1.2 Vascular Smooth Muscle

Similar mechanisms exist in vascular smooth muscle as in cardiac muscle for pH regulation (Aalkjaer 1990). As such, the Na^+-H^+ exchanger has been described in primary cultures of rat aortic smooth muscle cells (Weissberg *et al.,* 1987). Intracellular pH regulation has also been shown to be dependent on the physiological buffers used. In the A10 smooth muscle cell line using the intracellular pH-sensitive dye 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), it was shown that the Na^+-H^+ exchanger was responsible for >96% of pH recovery in the absence of HCO_3^-/CO_2 buffer. While a DIDS-sensitive Na^+-dependent HCO_3^--Cl^- exchanger was shown to contribute to the regulation in HCO_3^-/CO_2 buffer. At intracellular pH of 6.48 and 6.94, the Na^+-H^+ exchanger and DIDS-sensitive Na^+-dependent and -independent HCO_3^--Cl^- exchanger were shown to be the dominant mechanisms (Kikeri *et al.,* 1990). Similar results were obtained using the cell line BC3H-1 (Putnam 1990; Putnam *et al.,* 1990) and a primary culture of canine femoral artery (Kahn *et al.,* 1990). A study using cultured rat aortic smooth muscle cells calculated that HCO_3^--dependent mechanisms contribute to approximately 40% of recovery from a NH_4Cl pre-pulse (Little *et al.,* 1995).

Therefore, evidence exists that similar mechanisms are responsible for intracellular pH maintenance in cardiac and vascular smooth muscle cells. In addition, lactate and washout of CO_2 have been proposed as further pathways in cardiac myocytes

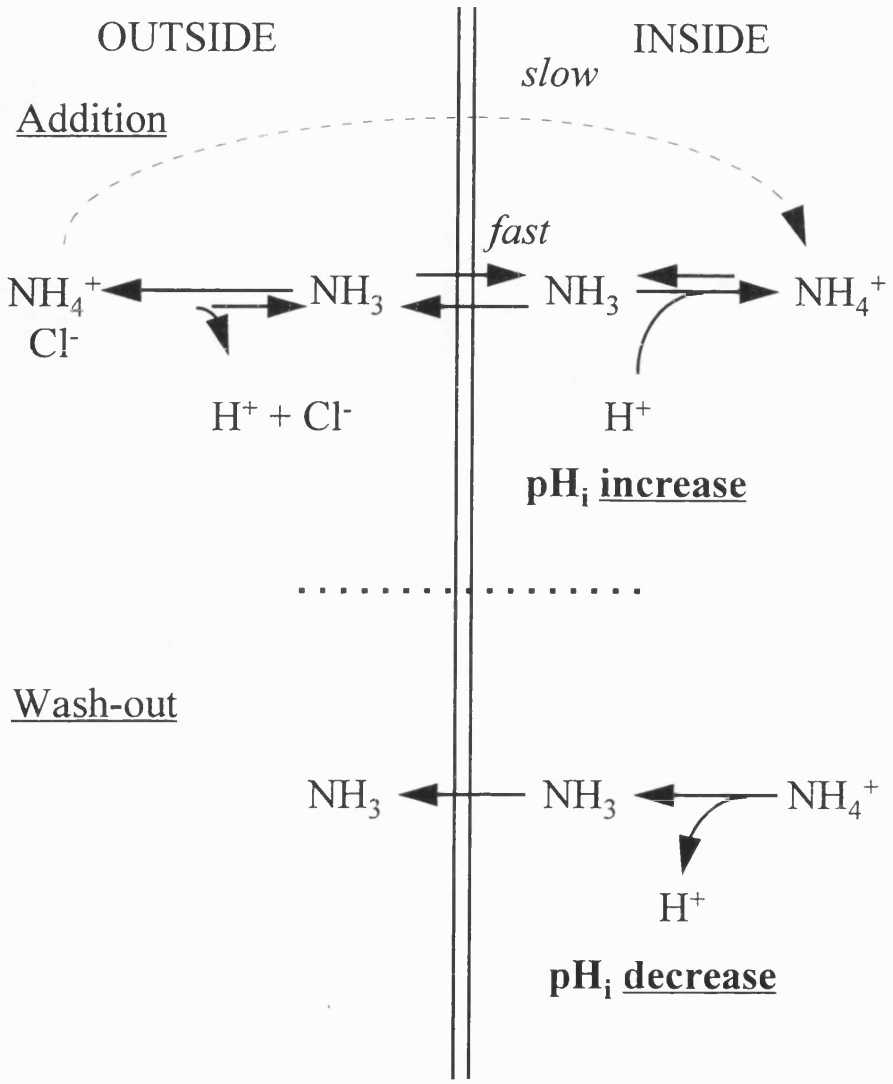


Figure I2: Representation of action of NH_4Cl on intracellular pH. As NH_3 enters the cell rapidly, it buffers intracellular protons, causing an alkaline shift in intracellular pH. On wash-out, NH_3 dissociates from the protons and diffuses out of the cell rapidly, causing an acid shift in intracellular pH.

1.2 Myocardial Ischaemia

Myocardial ischaemia is a condition where blood flow to the myocardium is insufficient to meet its demands, allowing the accumulation of metabolites, in addition to depriving the myocardium of substrate. Within cardiovascular disorders, myocardial ischaemia is the most costly both in human and economic terms. It occurs due to coronary thrombosis, coronary spasm, cardiac arrest, or atherosclerosis of the coronary arteries in the face of increased cardiac workload, and in clinical situations during a variety of surgical procedures *e.g.* balloon angioplasty (Pierce *et al.,* 1995; Kette *et al.,* 1993). Two main factors determine the damage occurring during ischaemia; these are the duration of the ischaemic insult and the degree of flow reduction. Longer periods are worse than transient events, likewise no-flow ischaemia, which rarely occurs clinically, is worse *c.f.* low flow (Applegate *et al.,* 1990).

Myocardial ischaemia manifests itself as a decreased cardiac contractility and is often associated with the generation of arrhythmias. The reduced blood flow not only deprives the heart of necessary substrates *i.e.* glucose, fatty acids and oxygen but also allows the accumulation of acidic metabolites and imbalances of vital ions.

1.2.1 Ionic Consequences Of Myocardial Ischaemia

Under normal circumstances the generation of adenosine tri-phosphate (ATP) is aerobic *i.e* requires oxygen. During ischaemia, however, the lack of substrates (glucose, fatty acids and oxygen) for this pathway means ATP generation becomes anaerobic. There is a fall in the concentration of phosphocreatine which precedes a marked fall in the

concentration of ATP (de Albuquerque *et al.,* 1994), while inorganic phosphate concentration increases (Smith *et al.,* 1993). In a study using rat hearts, tissue ATP levels were depleted following 20 minutes of ischaemia, this fall in tissue ATP levels is thought of as a key factor in the development in ischemic contracture which will be discussed later (Chen *et al.,* 1996).

Na^+, Ca^{2+}, K^+ and H^+ homeostasis are all disturbed during ischaemia. These ions normally exist below their electrochemical equilibriums, implying that active processes requiring ATP are responsible during normal homeostasis. Since ATP levels fall during ischaemia, the concentrations of these ions move down their concentration gradients (Pierce *et al.,* 1995).

The role of increases in intracellular Na^+ and ischaemia-induced damage is complex and involves several cellular processes. It is thought that the intracellular acidosis observed with ischaemia stimulates the Na^+-H^+ exchanger in an attempt to expel the protons. This increases intracellular Na^+, which in turn, will stimulate Na^+-Ca^{2+} exchange to increase intracellular Ca^{2+} (Avrikan *et al.,* 1992). A further factor thought to increase intracellular Na^+ during ischaemia is decreased Na^+-K^+ ATPase activity (which pumps Na^+ out and K^+ into the cell). Since ATP levels fall, not only will intracellular Na^+ rise but extracellular K^+ will also increase.

The movement of K^+ from inside to outside of cells during myocardial ischaemia is well established (Kleber 1984). Mechanisms responsible may include a reduction in Na^+-K^+ ATPase activity and an increase in K^+ efflux through ATP-sensitive K^+ channels (Pierce *et al.,* 1995). K^+ levels undergo a bi-phasic increase which is dependent on the duration

of the ischaemic period. There is an initial rise which increases within 15 seconds of the onset of ischaemia, until a plateau is reached ($[K^+] \approx 15mM$) 10 minutes later (Kleber 1984; Wilde & Aksnes 1995; Vermeulen *et.al.* 1996). Reperfusion, at this stage, leads to a rapid return of K^+ levels to normal. If, however, the ischaemic period is longer (greater than 20 minutes) extracellular K^+ levels rise further, possibly due to irreversible damage of the cell membrane.

Oxygen-derived free radicals have been shown to be generated during myocardial ischaemia. Production of the radicals begins during coronary occlusion, reaching a peak after 3 minutes, and may persist for several hours after re-perfusion (Bolli *et al.* 1988). Free radicals have been demonstrated to alter many characteristics of cardiac including depression of peak force without altering the Ca^{2+} sensitivity of skinned rat cardiac muscle (MacFarlane & Miller 1993). In addition, oxygen-derived free radicals have been shown to have specific effects on the electrical activity of cardiac cells. Jabr & Cole (1993) demonstrated that radicals caused specific alterations in membrane ionic currents, leading to changes in resting membrane potential and action potential configuration. Furthermore, radicals have also been shown to decrease the current which flows through L-type Ca^{2+} channels in guinea pig cardiac myocytes (Coetzee & Opie 1992).

1.3 *Myocardial pH During Ischaemia*

By using several experimental techniques, including nuclear magnetic resonance (NMR) and microelectrodes, myocardial intracellular pH has been estimated to be approximately 6.9 - 7.1 (Poole-Wilson 1978). This figure has been demonstrated to decrease to levels as low as pH 6.0 following the onset of ischaemia (Mohabir *et al.*, 1991; de Albuquerque *et al.*, 1994; Chen *et al.*, 1996). However, the origin of the protons which cause the acidosis in ischaemia has been disputed.

Generation of protons during normal metabolism can occur by several pathways, including those listed below:

$ATP \rightarrow ADP + Pi + nH^+$	$n = 0.8$
$PCr + nH^+ \rightarrow Cr + Pi$	$n = 0.4$
$glucose \rightarrow 2\ lactate + nH^+$	$n = 2.0$
(Allen *et al.*, 1987)	n = number of protons produced

A study using isolated ferret hearts and NMR has described that acidosis results from inhibition of aerobic respiration, as would be seen in hypoxia. Further to this, inhibition of glycolysis partially (2 hour exposure to glucose-free solutions) or fully (20 minute exposure to iodoacetate), demonstrated that the acidosis produced by cyanide is partly/fully due to protons released from ATP breakdown (Smith *et al.*, 1993).

However, Chen *et al.* (1996) questioned ATP breakdown as the source of protons during ischaemia in rat heart. They observed a decrease in intracellular pH during global ischaemia and a decrease in tissue ATP, but following a further decrease in tissue ATP, no further fall in intracellular pH was observed. Recent evidence has shown that the rate and extent of anaerobic glycolysis is the primary determinant of acidosis during ischaemia (Applegate *et al.,* 1990).

One further possible source of acidosis during ischaemia is CO_2 retention. In a porcine model of cardiac arrest where ventricular fibrillation was induced by delivery of alternating current to the epicardium (Kette *et al.,* 1993), intramyocardial pCO_2 increased from 54 torr to 346 torr, while pH fell from pH 7.2 ($[H^+]$ 65nmol H^+/L) to 6.38 ($[H^+]$ 441nmol H^+/L). Similar results were also obtained in a rat model of global ischaemia associated with cardiac arrest (Johnson *et al.,* 1995).

Therefore, the increase in proton concentration during myocardial ischaemia is well recognised, although the origin of the protons is controversial.

1.4 Cardiac Contractility

Myocardial contraction and, to a certain degree, its regulation can be understood as the interactions of six proteins (Table I1 and figure I3). These proteins exhibit prominent features of cardiac contraction. Namely, ATP hydrolysis, which in turn leads to tension development and the regulation by intracellular Ca^{2+}.

1.4.1 Contractile Proteins

Protein	Location	Properties
Myosin	Thick filament	ATP hydrolysis Actin interactions
Actin	Thin filament	Activates myosin ATPase Myosin interactions
Tropomyosin	Thin filament	Modulates actin-myosin interactions
Troponin C	Thin filament	Ca^{2+} binding
Troponin I	Thin filament	Inhibits actin-myosin interactions
Troponin T	Thin filament	Binds troponin complex to thin filament

Table I1: Proteins involved in cardiac contraction

Myosin is a large molecule which is the principal protein of the thick filament. A myosin molecule is comprised of a tail (which weave together to form the rigid backbone) and an active head which projects as the cross-bridge. It is within these heads that the ATPase characteristics are present. Myosin has another feature central to contraction which also resides in the molecules head, namely its ability to bind to actin.

Actin is smaller than myosin (MW ≈42kDa *cf.*480kDa) which exists as a slightly ovoid shape. It forms a double-stranded helix, each strand being a chain of actin monomers. It has a very important role in contraction, since it activates myosin ATPase activity. Within the molecule, actin provides the backbone of the thin filament.

Tropomyosin consists of two helical peptide chains linked by disulphide bridges, forming a relatively inflexible complex. Tropomyosin binds to actin along the groove in the actin double helix. Here, it adds the structure and rigidity of the thin filament and, through a series of interactions with other proteins plays a role in regulation of contraction.

The last three proteins exist within the troponin complex. Troponin C contains the calcium binding site that regulates contraction. Troponin I, acting with tropomyosin, regulates the interactions between actin and myosin. Troponin T binds troponin to tropomyosin.

1.4.2 Cardiac Contraction

Depolarization of myocardial cells results in influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels. This Ca^{2+} not only contributes to tension development, but will also cause Ca^{2+} to be released from the sarcoplasmic reticulum (Na^{+}-Ca^{2+} can also contribute to Ca^{2+} entry). This increase in intracellular Ca^{2+} is recognized by troponin C, reversing an inhibitory effect of regulatory proteins. This shift in tropomyosin allows the active sites of actin to bind to troponin, allowing the chemical processes of cardiac contraction to occur.

The contractile processes is summarized in figure I3. Myosin has a high affinity for ATP, such that virtually all myosin exists either bound to ATP or the products of its hydrolysis. This reaction energises the myosin, with the myosin complex being extremely stable. The dissociation, however, can be accelerated by the binding of actin. Release of ADP and inorganic phosphate from the actin myosin complex has the effect of "pulling" the attached thin filaments towards the centre of the sarcomere. This stage of a rigor complex now requires ATP to dissociate and the cross bridge cycling can be initiated again.

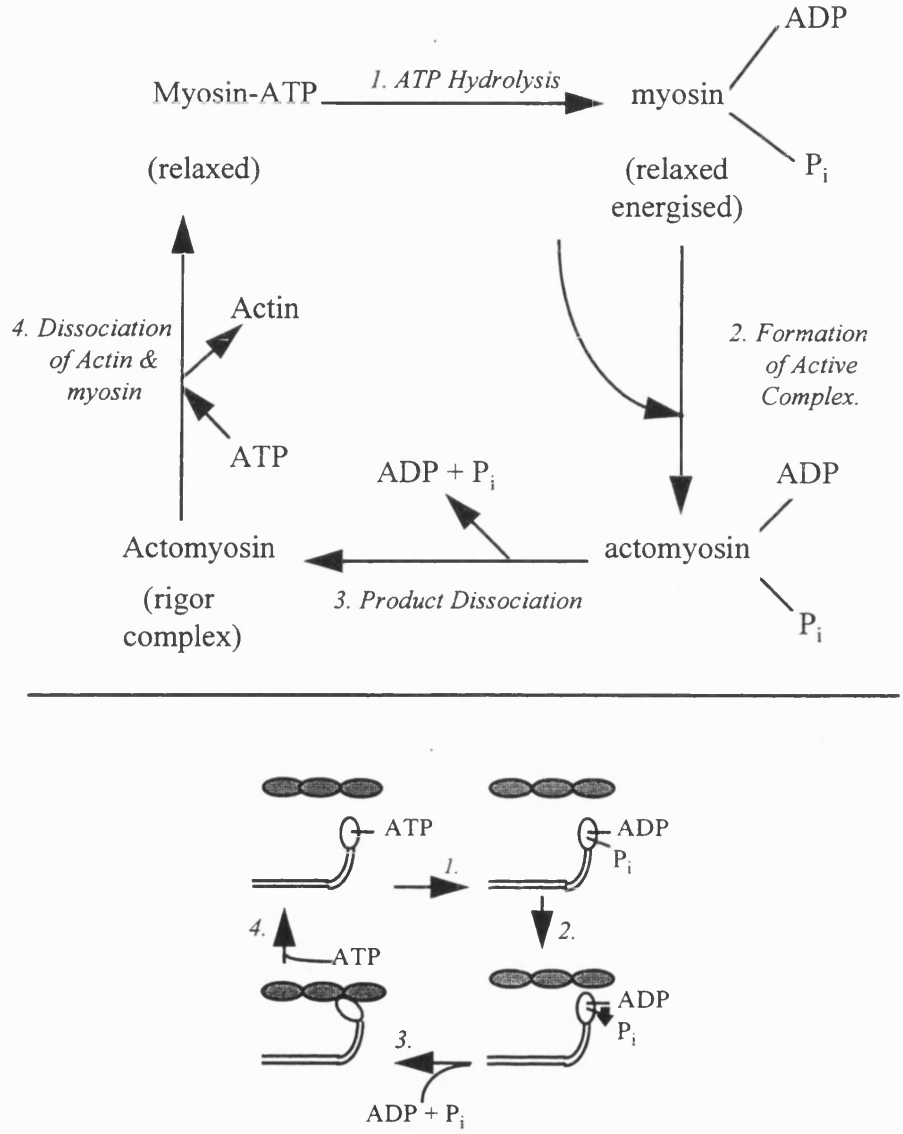


Figure I3: Diagram representation of cardiac contraction. Both panels describe the relationship between actin, myosin and ATP.

1.5 Modulation Of Cardiac Contractility

One of the prime methods whereby factors alter cardiac contractility is by alteration of the Ca^{2+} levels during systole. Normally, Ca^{2+} entering a myocyte through voltage-sensitive Ca^{2+} channels will have two main actions. A small amount will activate the contractile proteins as previously discussed. The vast majority, however, will cause further release of Ca^{2+} from the sarcoplasmic reticulum via an action at ryanodine receptors. This Ca^{2+} will cause further activation of the contractile proteins. Ca^{2+} is removed from the cytosol by the Na^{+}-Ca^{2+} exchanger and Ca^{2+} ATPase located on the sarcoplasmic reticulum and the cell surface.

1.5.1 Effects Of pH On Cardiac Contraction

Since the first reports in 1880, it is now well established that a decrease in pH has a profound effect on cardiac contraction. Increases in proton concentration can affect several steps in the process of excitation-contraction (Orchard & Kentish 1990). These include:

1.5.1.1 Ca^{2+} Concentration And Influx

Aequorin, a Ca^{2+}-sensitive photoprotein, was used to study the changes in intracellular Ca^{2+} in cat, ferret, rabbit and rat papillary muscle in response to pH changes. Tension was also measured (Allen *et al.,* 1983). It was demonstrated that increasing the CO_2 concentration of the extracellular fluid from 5 to 15% (pH change of 7.36 to 6.91),

decreased force in all species studied. Surprisingly, intracellular Ca^{2+} increased to a steady-state above basal. Similar effects were also observed using FURA-2, another intracellular dye sensitive to Ca^{2+} (Nakanishi *et al.,* 1990). Increasing Ca^{2+} in cultured ventricular myocytes using the NH_4Cl pre-pulse technique was shown to be due to changes in intracellular Ca^{2+} buffering and Ca^{2+} extrusion as opposed to Ca^{2+} influx (Kim *et al.,* 1987; Kohomoto *et al.,* 1990).

Mohabir *et al.* (1991) utilised two intracellular dyes, 1-[2-Amino-5-(6-carboxyindol-2-yl)-2-(2'-amino-5'-methylphenoxy)ethane-N,N,N,N,-tetraacetic acid pentaacetoxy-methyl -ester (INDO-1) for Ca^{2+} measurements and BCECF for pH measurements in isolated perfused rabbit hearts where recordings were made at the epicardial surface of the left ventricle. Global ischaemia decreased contractility and increased end systolic and diastolic Ca^{2+} levels. Concomitantly intracellular pH decreased from 7.03±0.06 to 6.11±0.04 after 15 minutes. There is evidence that this increase in cytosolic Ca^{2+} level during acidosis may occur through a disturbance of Ca^{2+} handling of the sarcoplasmic reticulum since the acidosis-induced increase was attenuated by ryanodine (Orchard 1987).

Acidosis also has an inhibitory effect on Ca^{2+} currents flowing through voltage-sensitive Ca^{2+} channels. In a study using isolated guinea-pig ventricular myocytes and with Ba^{2+} as the charge carrier, it was demonstrated that an increase in intracellular protons reduced the current through voltage-sensitive Ca^{2+} channels. In the same study, it was revealed that the protons shifted the activation curve of these channels in a rightward (negative) direction by 10-15mV (Kaibara *et al.,* 1988).

1.5.1.2 Force Production

There is a sigmoidal relationship between force and intracellular Ca^{2+}. By performing Ca^{2+}-contraction curves at acidic pH, alterations in contractility can be observed. Changes in pH have a dramatic effect on this relationship in cardiac muscle (Kentish *et al.* 1994). By normalising the maximum activated force to unity, two types of alterations are revealed. Since Ca^{2+} levels in the cell are increased during acidosis, this would suggest that acidosis is reducing the responsiveness of the contractile proteins to Ca^{2+}.

Experiments performed using ferret hearts showed that CO_2-induced acidosis caused a decrease in twitch tension which was produced primarily by shifting myofilament Ca^{2+} sensitivity to the left (Marban *et al.,* 1987). Similar observations were obtained using ferret papillary muscle. This study also showed that the decreased force produced in response to acidosis could be reversed by increasing the Ca^{2+} concentration of the bathing solution (Orchard *et al.,* 1991). In further studies on rat atria and skinned ventricular preparations (Palmer *et al.,* 1996), this reduction in myofibrillar sensitivity was calculated to be 1.09pCa and 1.04pCa units respectively (where $pCa = -\log_{10}[Ca^{2+}]$). The mechanism responsible for reducing myofibrillar sensitivity to Ca^{2+} is thought to be due to a decrease in Ca^{2+} binding to its regulatory site on troponin C.

Acidosis (pH 6.5) has been shown to inhibit myosin ATPase activity in canine myofibrils. The concentration of Ca^{2+} required for 50% activation increased by 0.4 pCa unit (Solaro *et al.,* 1986). The stiffness of skinned cardiac muscle, a measure of number of myosin cross-bridges attached to myosin, has also been shown to be reduced in acidic conditions. This would suggest fewer attached cross-bridges. The fall in stiffness is

proportionately less than the decrease in force, implying that there is a reduction in the mean force produced per cross-bridge (Fabiato *et al.,* 1978).

This reduction in force is amplified at submaximal cytosolic Ca^{2+} concentrations. Taken with the inhibitory effect of acidosis on myosin ATPase rates, acidosis reduces the efficiency of muscle contraction by reducing the force produced per ATP molecule hydrolysed.

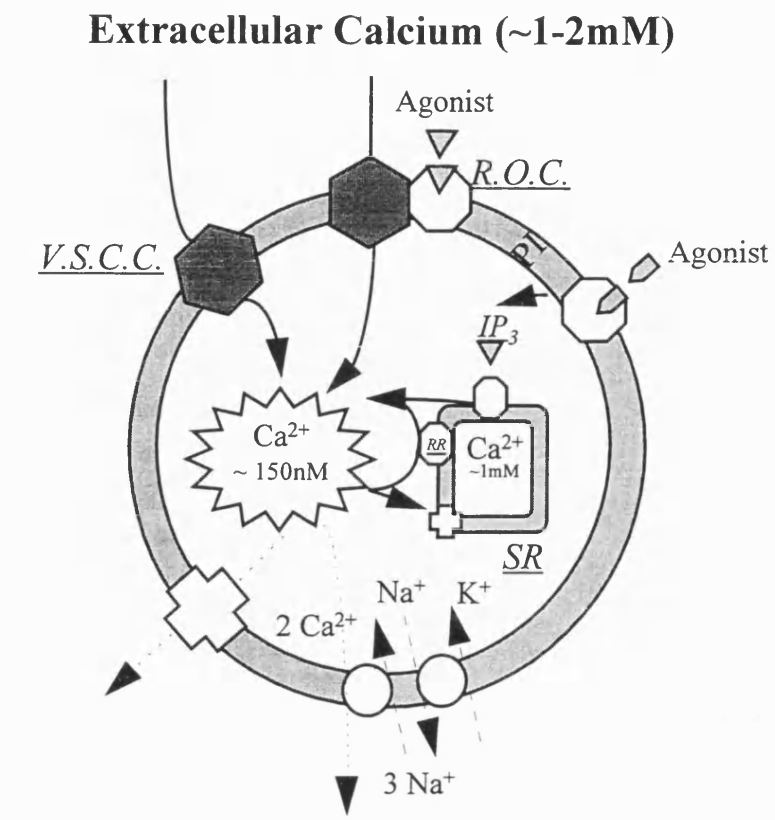
1.6 Ca^{2+} And Smooth Muscle Contraction

Contraction of vascular smooth muscle is largely, but not entirely, dependent on the concentration of free Ca^{2+} within the cytosol. This, in turn, is under the control of various channels and pumps located in the sarcolemma and the sarcoplasmic reticulum (SR); the cells main source of intracellular Ca^{2+}. Between these mechanisms, intracellular free Ca^{2+} concentration is tightly regulated between 0.1-0.3μM. Following stimulation, *e.g.* agonist occupancy, depolarization *etc.*, Ca^{2+} enters the cell from the extracellular fluid or is released from the SR. In both cases, Ca^{2+} diffuses down its concentration gradient since Ca^{2+} concentration of both the extracellular fluid and SR is in the mM range.

1.6.1 Ca^{2+} Homeostasis

In the resting state, intracellular Ca^{2+} concentration is tightly regulated by various mechanisms at approximately 0.1 to 0.3μM. Some of these processes include mechanisms whereby Ca^{2+} is either extruded from the cytoplasm or taken up by cell organelles (Himpens *et al.* 1995; Orallo 1996).

There is a protein located on the cell membrane that exchanges Ca^{2+} ions for protons. This is termed the Ca^{2+} pump or the plasmalemmal Ca^{2+}-ATPase, as it uses ATP to drive the process (see figure I4). It has low capacity but a high affinity for Ca^{2+}. Ca^{2+} can also be extruded across the cell membrane by high capacity, but low affinity ionic exchange, with one Ca^{2+} ion being exchanged for three Na^{+} ions. Between the two systems, both fine control (exerted by Ca^{2+}-ATPase) and bulk control (Na^{+}-Ca^{2+} exchange) can be achieved by their different affinities and capacities.



Key:

Ca²⁺ATPase

Ion exchanger

Ion channel

Receptor

IP₃ Inositol 1, 4, 5-trisphosphate

ROC Receptor operated channel

SR Sarcoplasmic reticulum

VSCC Voltage sensitive Ca^{2+} channel

RR Ryanodine receptor

Figure I4: Schematic representation of Ca^{2+} handling within a vascular smooth muscle cell.

Regulation of intracellular Ca^{2+} is also provided by Ca^{2+} transport across intracellular membranes. The mitochondria can play a role in Ca^{2+} regulation, with Ca^{2+} entry being closely linked to proton production via oxidative phosphorylation. Under normal circumstances, however, it is thought that this route has a minor role, becoming important when Ca^{2+} levels become pathologically high. A further Ca^{2+} sink/store that may participate is the nucleus. The major intracellular store which has an important role in homeostasis is the sarcoplasmic reticulum. Like the plasmalemma, the sarcoplasmic membrane contains Na^{+}-Ca^{2+} exchangers, Ca^{2+}-ATPase and Ca^{2+} channels (ryanodine and IP_3 receptors; van Breemen *et al.,* 1989).

1.6.2 Mechanisms of Increasing Intracellular Ca^{2+}

As mentioned above, contraction of vascular smooth muscle is highly dependent on the concentration of cytoplasmic Ca^{2+}. Intracellular Ca^{2+} is required for many cellular processes and changes in the concentration of intracellular Ca^{2+} play an important role in excitation-contraction in vascular smooth muscle.

Ca^{2+} channels can be subdivided into those whose opening probability is increased under conditions of membrane depolarization and those primarily controlled by ligand binding to appropriate receptors. One of the principal routes employed by vascular smooth muscle cells to increase cytoplasmic Ca^{2+} is via influx through these voltage-sensitive Ca^{2+} channels located in the plasma membrane. It is possible, however, to increase intracellular Ca^{2+} independent of changes in membrane potential (see figure I5).

Pharmacomechanical coupling describes a chain of events where contraction occurs independent of changes in the membrane potential of the vascular smooth muscle cell. This increase in intracellular Ca^{2+} occurs via changes in intracellular second messengers, which are important mediators of both contraction and relaxation in vascular smooth muscle. They include primarily, cytosolic free Ca^{2+}, inositol- 1, 4, 5, -trisphosphate (IP_3) which can also increase Ca^{2+} levels due to release of Ca^{2+} from the sarcoplasmic reticulum. When intracellular Ca^{2+} has increased, Ca^{2+} can cause contraction, or, can release further Ca^{2+} from the SR, a phenomenon known as Calcium Induced Calcium Release (CICR).

Figure I5: Representation of mechanism of initiation of contraction and relaxation in smooth muscle cells (Rang & Dale 1992).

1.7 Voltage-Sensitive Ca^{2+} Channels

Three types of voltage-sensitive Ca^{2+} channels have been demonstrated in a variety of vascular preparations. They are the R-type channel, the transient, low threshold-activated T-type channel and the sustained, high threshold L-type Ca^{2+} channel. It is the latter which has the key role in membrane depolarization and Ca^{2+} entry (Nelson *et al.*, 1990; Hughes 1995).

The physiological role for the R-type Ca^{2+} channel, reported in renal vessels, is still not clear. A study using the arterial and venous vasculature of the double-perfused rat mesenteric bed implicated the R-type channel in the arterial vasodilation and venoconstriction observed with platelet-activating factor. Interestingly, the same study described differences in sensitivities of this venoconstriction to two Ca^{2+} channel antagonists. Isradipine could inhibit the responses on both arterial and venous circulations (whilst having no effect on dilations to acetylcholine and constrictions to angiotensin II), while nifedipine could not (Claing *et al.*, 1994). It is thought that it may be play a pivotal role in Ca^{2+} entry during tonic contractions observed in vascular preparations when depolarization is induced by high K^+ solutions.

Ca^{2+} influx through T-type Ca^{2+} channels has been demonstrated in guinea-pig mesenteric artery vascular smooth muscle. But it is doubtful that at the holding potential of -80mV and the disappearance of the current at -40mV, along with rapid inactivation (20-60msec) that this would be significant in Ca^{2+} entry, at least in relation to contraction in a normal cell at physiological membrane potentials.

L-type Ca^{2+} channels have been demonstrated to be different to T-type channels in many respects (Xiong & Sperelakis 1995). L-type Ca^{2+} channels have a high threshold level (-35mV *cf.* -60mV for T-type channel) and a high single channel conductance (18-26pS *cf.* 8-12pS). L-type Ca^{2+} channels are highly sensitive to dihydropyridine Ca^{2+} antagonists, whereas these drugs have only a modest effect on T-type channels. The Ca^{2+} channel agonist, BAY-K-6844, is ineffective at T-type channels but causes a leftward shift in the activation curve for L-type Ca^{2+} channels.

The L-type Ca^{2+} channel located in the cell membrane is a major pathway for Ca^{2+} entry during excitation and regulation of the force of contraction of the muscle (Nelson *et al.,* 1990). In addition, the current is steeply dependent on membrane potential. Since the resting membrane potential of a vascular smooth muscle cell lies in the region -40 to -60 mV, it is reasonable to assume that there could be a fraction of L-type Ca^{2+} channels open at rest. With such a high degree of dependency on membrane potential, this would mean small changes in membrane potential would be expected to cause large changes in Ca^{2+} influx and hence alterations in the contractile state of the cell.

Opening of voltage sensitive Ca^{2+} channels has been shown to be facilitated by noradrenaline in rabbit mesenteric arteries (Nelson *et al.,* 1988). Application of noradrenaline increased the open probability of the channels at physiological membrane potentials.

These voltage-sensitive channels have also been proposed to play a key role in stretch-induced contraction, whereby an increase in intravascular pressure stretches the smooth muscle cell which contracts in response (Meininger & Davis 1992; Wesselman *et al.*, 1996).

1.8 Receptor Operated Ca^{2+} Channels

This classification of ion channels differ from the voltage-sensitive channels as they are not sensitive to fluctuations in membrane potential. Instead, when an agonist binds to its respective receptor, it allows the rapid flow of ions down their electrochemical gradient. Evidence for the existence of such channels is derived from experiments investigating Ca^{2+} influx and tone (Nishimura *et al.*, 1989). It was demonstrated that noradrenaline could increase $^{45}Ca^{2+}$ influx and force without causing membrane depolarization. Similar increases could also be observed in depolarized aortic preparations.

1.8.1 Generation Of IP_3 And DAG

As described in figure I5, the interactions of receptors located on the cell surface, G proteins and phospholipase C (PLC) results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate. This reaction releases two main factors, water soluble IP_3 and lipid soluble 1,2-diacylglycerol (DAG) (Nahorski *et al.*, 1994).

There are many membrane receptors capable of stimulating phosphoinositide turnover via the PLC enzyme family of which subtypes exist. Briefly, the receptors which utilise this signalling pathway are generally characterised by having seven transmembrane

domains. These domains interact with each other to form an agonist binding region which, when activated, confers a conformational change responsible for activating the respective G protein. For example, activation of α-adrenergic receptors stimulates PLCβ (of which further subtypes exist) via stimulation of the G-protein, G_q (Berridge 1993).

IP_3 is recognized as an intracellular messenger which, upon interaction with specific IP_3 receptors, is capable of releasing intracellular Ca^{2+} stores. IP_3 receptors can be distinguished from other intracellular receptors, namely ryanodine receptors, by their relative insensitivity to ryanodine, caffeine or ruthenium red (Table I2). They are, however, blocked by heparin. They have a low conductance (20pS) and are voltage-independent (Joseph 1992). There is now a family of IP_3 receptors, with diversity arising due to alternative splicing and existence of separate genes. These channels are also distributed differently in tissues and cell lines (Mikoshiba 1993).

Noradrenaline has been demonstrated to generate IP_3 in saponin-treated rabbit mesenteric arterial smooth muscle, leading to contraction (Itoh *et al.,* 1992). Such increases in IP_3, however, are transient returning to baseline levels within 30 seconds. This increase in IP_3 is associated with an initial increase in cytoplasmic Ca^{2+} and a phasic contraction (Nahorski *et al.,* 1994). IP_3-induced Ca^{2+} release has been demonstrated to be pH-sensitive. In saponin-treated guinea-pig portal vein smooth muscle cells, increases in intracellular pH increased the rate of IP_3-induced calcium release whereas decreases in pH reduced it. Such responses may occur by protons inhibiting the binding of IP_3 to its receptor (Tsukioka *et al.,* 1994).

The other product of PLC-induced phosphoinositide hydrolysis is 1,2-diacylglycerol. This substance is the endogenous activator of protein kinase C. This, in turn, has been demonstrated to activate the Na^+-H^+ exchanger and cause intracellular alkalosis (Rasmussen *et al.,* 1987). This could potentially alter the sensitivity of the contractile apparatus with respect to Ca^{2+}.

Property	IP_3 receptor	Ryanodine receptor
Structure	homotetrameric	homotetrameric
Molecular weight	4 x 313kDa	4 x 565kDa
Physiological activators	IP_3	Ca^{2+} cADP ribose (?)
a) Ca^{2+}	+ <0.3μM - >0.3μM	+ <100μM - > 1mM
b) ATP	+ <2mM - >4mM	+ mM
Pharmacological activators	Enantiomers of IP_3	caffeine (5mM) heparin (2μg.ml^{-1}) ryanodine (<10μM)
Pharmacological inhibitors	heparin (10μg.ml^{-1}) caffeine (10mM)	ruthenium red (10nM) ryanodine (>10μM) Mg^{2+} (mM)

+ increases activity - decreases activity

Table I2: Comparison of IP_3 and ryanodine receptors with respect to structure, size and various activators and inhibitors.

\+ Positive effect on calcium levels

\- Negative effect on calcium levels (Ehrlich *et al.*, 1994)

Ca^{2+} mobilisation can also occur via a mechanism independent of the generation of IP_3, this is termed Calcium Induced Calcium Release (CICR). CICR appears to occur via receptors which share a large amount of structural homology with IP_3. Ca^{2+} itself (<100μM) is one of the triggers for release of further Ca^{2+} from the SR provided there is sufficient calcium in storage (see Table 3 and figure I2). This effect is mediated by ryanodine receptors.

Like IP_3 receptors, ryanodine receptors are distributed differently around tissues. Ryanodine receptor 1 is found in rabbit skeletal muscle, heart and smooth muscle. Ryanodine receptor 2 is located in rabbit and bovine heart, plus guinea pig, hamster and rat ventricle. Ryanodine receptor 3 is found in a variety of rabbit brain regions, plus rabbit aorta, urinary bladder ureter (Coronado *et al.,* 1994). Ryanodine (>10μM), in addition to causing Ca^{2+} release, has been demonstrated to inhibit the sarcolemmal Na^+-Ca^{2+} exchanger (Wagner-Mann *et al.,* 1992), possibly contributing to the observed increase in intracellular Ca^{2+}. The Ca^{2+} released by ryanodine is thought to be functionally separate from IP_3 sensitive pools. This has been demonstrated in a number of vascular preparations including rat aorta (Kanaide *et al.,* 1987), rat mesenteric artery (Baro *et al.,* 1992) and cultured rat arterial vascular smooth muscle cells (Tribe *et al.,* 1994).

More recently, a putative endogenous activator of the ryanodine receptor has been investigated. The NAD^+ metabolite, cyclic ADP ribose (cADPR), has been demonstrated to release intracellular Ca^{2+} from an IP_3 insensitive pool (Kannan *et al.,* 1996). This Ca^{2+} release was desensitized to subsequent applications of cADPR, caffeine and ryanodine but not IP_3.

Therefore, the two distinct mechanisms (IP_3 and ryanodine receptors) form a powerful amplification pathway for Ca^{2+} entry leading to contraction. IP_3 has been put forward as the major intracellular messenger concerned with Ca^{2+} release in vascular smooth muscle. However, the ability of cADPR, to cause Ca^{2+} release from smooth muscle suggests that ryanodine-induced release of Ca^{2+} may also be important in the vasculature (Kannan *et al.,* 1996).

There is, however, another putative pathway that utilises an intracellular molecule to raise intracellular Ca^{2+}, namely the Ca^{2+} influx pathway. The role of the Ca^{2+} influx pathway is not yet substantiated in vascular smooth muscle. This describes a process by which intracellular Ca^{2+} increases in response to stimuli, without any discernible increase in recognised second messengers. Ca^{2+} depletion of a lymphocyte cell line (Jurkhat) caused a soluble second messenger (Calcium Influx Factor; CIF) to be released from intracellular organelles. When applied to other cell types, CIF caused Ca^{2+} influx (Randriamampita *et al.,* 1993). A small (<500D) phosphate-containing anion purified from cellular subfractionation has been demonstrated to have similar properties as CIF (Fasolato *et al.,* 1994). It is now thought that CIF could be the signal used by cells to cause Ca^{2+} influx to possibly replenish reduced intracellular stores. There is no doubt cast over the existence of CIF (Clapham 1995)

A further mechanism put forward as a possible mechanism for capacitative Ca^{2+} entry is that store depletion confers a conformational change in dihydropyridine receptors and ryanodine receptors (Berridge 1997).

IP_3 and ryanodine receptors are both located on the sarcoplasmic reticulum of vascular smooth muscle. Until recently, the sarcoplasmic reticulum was thought of as a single store for Ca^{2+} release. Now, it is functionally split into two sections. This is known as the "superficial sarcoplasmic buffer barrier" hypothesis (van Breemen *et al.,* 1995).

As mentioned above, the superficial sarcoplasmic buffer barrier is thought to play a role in Ca^{2+} homeostasis. The plasma and sarcoplasmic membranes are extremely physically close, the distance between them being 10-100nm. At rest, a significant proportion of calcium entering the cell is pumped into the sarcoplasmic reticulum before it can contribute to contraction. When stimulated by PLC-linked receptors, this pathway is "by-passed" and the vast majority of Ca^{2+} entering contributes to contraction. The Ca^{2+}-ATPase of the sarcoplasmic reticulum located deeper contributes to Ca^{2+} extrusion from the cytoplasm as it removes Ca^{2+} into its interior before it is pumped out of the cell via the plasmalemmal Na^{+}-Ca^{2+} exchanger (Chen *et al.,* 1993).

The above section describes various ways in which an agonist may increase intracellular Ca^{2+}. In reality, it is doubtful whether any agonist acts via a single method. Instead, agonists are more likely to activate complex signal transduction mechanisms through multiple synergistic pathways to increase Ca^{2+}.

1.9 *Mechanics Of Ca^{2+} Dependent Smooth Muscle Contraction*

Cytoplasmic Ca^{2+} levels at rest are approximately 100-250nM. When stimulated this figure only rises transiently to 500-700nM, this small increase, however, is sufficient to cause contraction. The two main sources responsible for this increase in Ca^{2+} are the extracellular fluid and sarcoplasmic reticulum. The previous section described pathways within vascular smooth muscle that stimuli utilise to increase the cytoplasmic Ca^{2+} concentration. The next section will focus on the actions of intracellular with respect to contraction.

One of the most popular models for Ca^{2+} dependent contraction of smooth muscle is the cross-bridge cycling model. In this, contraction uses the thick and thin filaments, myosin and actin respectively, which slide over each other. The rise in sarcoplasmic Ca^{2+} is detected by calmodulin, a Ca^{2+} binding protein. Calmodulin binds Ca^{2+} which confers a structural change in the complex which results in the exposure of interactive sites for target proteins. In terms of smooth muscle contraction this target is myosin light chain kinase (MLCK), an enzyme with high affinity for the calmodulin-Ca^{2+} complex. The binding of the calmodulin complex converts the MLCK from an inactive to an active state. This then hydrolyses ATP and phosphorylates myosin. The phosphorylated myosin joins with actin to form phosphorylated actomyosin. This complex hydrolyses a further ATP molecule, developing force. This model is generally accepted to be the most important under normal physiological conditions (Walsh 1990; Somlyo *et al.*, 1994). A caveat to this is that smooth muscle contraction, though highly

dependent on intracellular Ca^{2+} and myosin phosphorylation, can occur at relatively low levels of these two variables.

1.9.1 Further Mechanisms Of Contraction

It has been demonstrated that Ca^{2+} and force development follow one of three patterns (see review by Morgan *et al.,* 1990):

1. Ca^{2+} levels can mirror force development exactly, both rising simultaneously to plateau levels, often observed with depolarized tissues. Observed with rat mesenteric vessels.

2. Force still increases to the same level but intracellular calcium displays only a transient increase, returning to baseline levels while contraction is maintained. Often seen with α adrenoceptor stimulation on rat mesenteric vessels.(Morgan *et al.,* 1984).

3. There is no measurable increase in calcium but force development is increased. Such a profile is seen with prostaglandin $F_{2\alpha}$ acting on porcine arteries and α agonist phenylephrine acting on ferret aorta (Suematsu *et al.,* 1991).

The existence of some dissociation between force and intracellular Ca^{2+} has been recognised as potentially implicating a second contractile pathway.

One such model for this calcium-independent contraction is the latchbridge hypothesis (Hai *et al.,* 1989). Within this, a contraction less reliant on Ca^{2+} occurs, possibly during sustained contractions after a rapid, transient, increase in calcium levels induced by agonists. This is followed by the subsequent restoration of resting levels of both Ca^{2+} and MLC phosphorylation. Briefly, the latchbridge state is associated with dephosphorylated attached crossbridges, with a decreased detachment rate. Myosin undergoes Ca^{2+} dependent phosphorylation to form phosphorylated myosin, the only Ca^{2+} dependent mechanism involved. This binds to actin to form an actin-myosin complex, similar to cycling crossbridges in Ca^{2+}-dependent contraction. This is then dephosphorylated to form the actin-myosin interaction that represents the latchbridge.

1.9.2 Caldesmon And Calponin

A scheme similar to the latchkey hypothesis has been proposed for maintenance of contraction in conditions of low Ca^{2+} (conditions 2 & 3; see above). Caldesmon, a 145kDa protein that can bind actin independently of Ca^{2+} has been proposed to act in a similar fashion to troponin in striated muscle. It binds actin in the absence of Ca^{2+} inhibiting actin-myosin interactions. In the presence of Ca^{2+}, however, caldesmon can bind to calmodulin, thus removing its inhibitory effect. Caldesmon can also bind myosin in the absence of Ca^{2+} and calmodulin, an interaction which is reversed in their presence. This linkage could infer a role for caldesmon cross-linking actin and myosin in tone maintenance during reduced Ca^{2+} levels (Adam *et al.,* 1989).

Caldesmon has another inhibitory role in contraction involving the inhibition of actin-myosin ATPase, abrogating cross-bridge cycling. Katsuyama *et al.* (1992) demonstrated that a fragment of caldesmon corresponding to the actin binding domain, but lacking the actin-myosin ATPase inhibitory region, could compete with endogenous caldesmon and cause contraction. In addition, this contraction was inhibited by increasing Ca^{2+} and calmodulin concentrations. Therefore, this would suggest that caldesmon is an endogenous inhibitor of smooth muscle contraction and a further possible mechanism for Ca^{2+}-independent contraction.

Another protein associated with actin and myosin is calponin which can bind actin in the absence/presence of Ca^{2+} although it requires Ca^{2+} to bind to calmodulin. It is thought to play a role similar to tropomyosin T, with which it shares some homology. In addition it has been shown to have actin-myosin ATPase inhibitory properties (Winder *et al.*, 1990; Carmichael *et al.*, 1994).

1.10 Smooth Muscle Relaxation

Relaxation of vascular smooth muscle can be mediated by several mechanisms through direct interference with any of the mechanisms involved in smooth muscle contraction. This could include reduction in Ca^{2+} entry, hyperpolarization of the cell membrane, or alterations in the concentration of the cytoplasmic second messengers. This could consist of a decrease in the concentration of IP_3 and DAG, or an increase in the vasodilatory mediators cyclic adenosine and cyclic guanine monophosphate (cAMP and cGMP respectively; McDaniel *et al.*, 1994)

A decrease in Ca^{2+} influx or the amount of Ca^{2+} released will reduce, in conjunction with Ca^{2+} efflux pathways, cytoplasmic Ca^{2+} concentration. Possible mechanisms include reduction of Ca^{2+} entry through voltage sensitive Ca^{2+} channels by specific antagonists *e.g.* dihydropyridines, or membrane hyperpolarization by activation of one/more K^+ channel(s) (Nelson *et al.,* 1995). Increased efflux of Ca^{2+} will restore the concentration of this ion to basal levels. As such, Na^+- Ca^{2+} exchange, in concert with Ca^{2+}ATPases, will move Ca^{2+} into the SR and subsequently out of the cell.

Reduction in intracellular Ca^{2+} levels, by either of the two methods above, will cause the contractile state to reverse. When the Ca^{2+} concentration is returned to basal levels, the calmodulin-MLCK complex is dissociated. MLC is dephosphorylated by MLC phosphatase and induce relaxation occurs (Somlyo *et al.,* 1994).

Relaxation of smooth muscle can also be affected by second messengers. One of these is endothelium derived relaxing factor (EDRF), or NO. Since the demonstration that acetylcholine-induced relaxations needed the presence of endothelial cells (Furchgott *et al.,* 1980), it is now recognised that NO is a labile molecule which is being constantly released from the endothelial layer.

For the purpose of relaxation of smooth muscle, the actions of NO are linked with the activation of cytosolic guanylate cyclase and the subsequent formation of cyclic guanine monophosphate (cGMP) (Rapoport & Murad 1983; Schulz *et al.,* 1994). cGMP has the ability to relax smooth muscle by several mechanisms, culminating in decreases in cytosolic Ca^{2+} levels and increases in myosin phosphorylation (Komalavilas & Lincoln 1996).

Another molecule which has the ability to relax smooth muscle is cyclic adenosine monophosphate (cAMP). For example, following stimulation of β adrenoceptors, the enzyme adenylate cyclase is stimulated to produce cAMP. This then stimulates a specific protein kinase, PKA, which phosphorylates and inactivates MLCK (Gerthoffer *et al.* 1984).

Discrepancies between the actions of acetylcholine on the membrane potential of rat mesenteric smooth muscle and the inability of a NO synthase inhibitor to attenuate the dilation has led to the proposition that the endothelial layer may produce a further entity capable of affecting smooth muscle cells (Garland & McPherson 1992). To this end, endothelial derived hyperpolarizing factor (EDHF) was proposed to exist. One possibility that has been proposed is that EDHF-like materials have been shown to be released in the isolated rat heart by bradykinin. It was postulated that this factor was a cytochrome P450 derived arachidonic acid metabolite (see review by Garland *et al.*, 1995).

EDHF is thought to mediate its effects by increasing membrane conductance, primarily of potassium ions, independent of the ATP-sensitive channel This channel has been shown to be activated in NO mediated hyperpolarizations (Murphy & Brayden 1995a). Contrary to this, glibenclamide was demonstrated to be ineffective against NO-independent hyperpolarizations (Garland & McPherson 1992). Evidence obtained in porcine tracheal smooth muscle cells suggests that NO can also act via another K^+ channel, the Ca^{2+}-activated K^+ channel (Yamakage *et al.* 1996). The same class of K^+ channel is thought to be involved in the action of EDHF (Murphy & Brayden 1995 b).

1.11 K^+ Channels in Smooth Muscle Cells

By allowing cells to confer selective permeability of a variety of ions, transmembrane ionic channels enable cells to establish electrical potentials across their cell membrane. This is an efficient mechanism whereby electrical signals can be transformed into cellular processes *e.g.* Ca^{2+} entry. This process of Ca^{2+} entry is largely governed by the membrane potential of the cell.

1.11.1 Resting Membrane Potential

If one assumes K^+ concentrations of $[K^+]_o$ 5 mM and $[K^+]_i$ 130-140mM, then the Nernst Equation at 37°C:

$$E_K = 61mV \log_{10} ([K^+]_o/[K^+]_i)$$

would give an equilibrium potential for K^+ (E_K) would be approximately -80 to -85mV. At any particular membrane potential, the driving force on K^+ ions is given by the relationship, membrane potential - E_K.

Since the membrane potential lies positive to E_K, the driving force will mean that K^+ will leave the cell. This K^+ efflux will shift the membrane potential more negative (hyperpolarization), making it more difficult to shift the membrane potential away from E_K. Since the K^+ equilibrium potential is so negative, K^+ channels tend to stabilize the membrane potential, holding the membrane potential at a negative value (Knot *et al.*,

1995). When similar calculations are performed for Ca^{2+} and Na^{+}, it is found when stimulated, these ions enter the cell causing depolarization.

Since the resting membrane potential of vascular smooth muscle cells (-40 to -60mV depending on tissue and experimental conditions) is more positive compared to E_K, K^{+} permeability is not the only ion involved. One other ion which may play a minor role in the resting membrane potential is Cl^{-}. With concentrations of $[Cl^{-}]_o$ 130mM and $[Cl^{-}]_i$ 15mM and a relatively high permeability, this could explain the discrepancy between E_K and membrane potential.

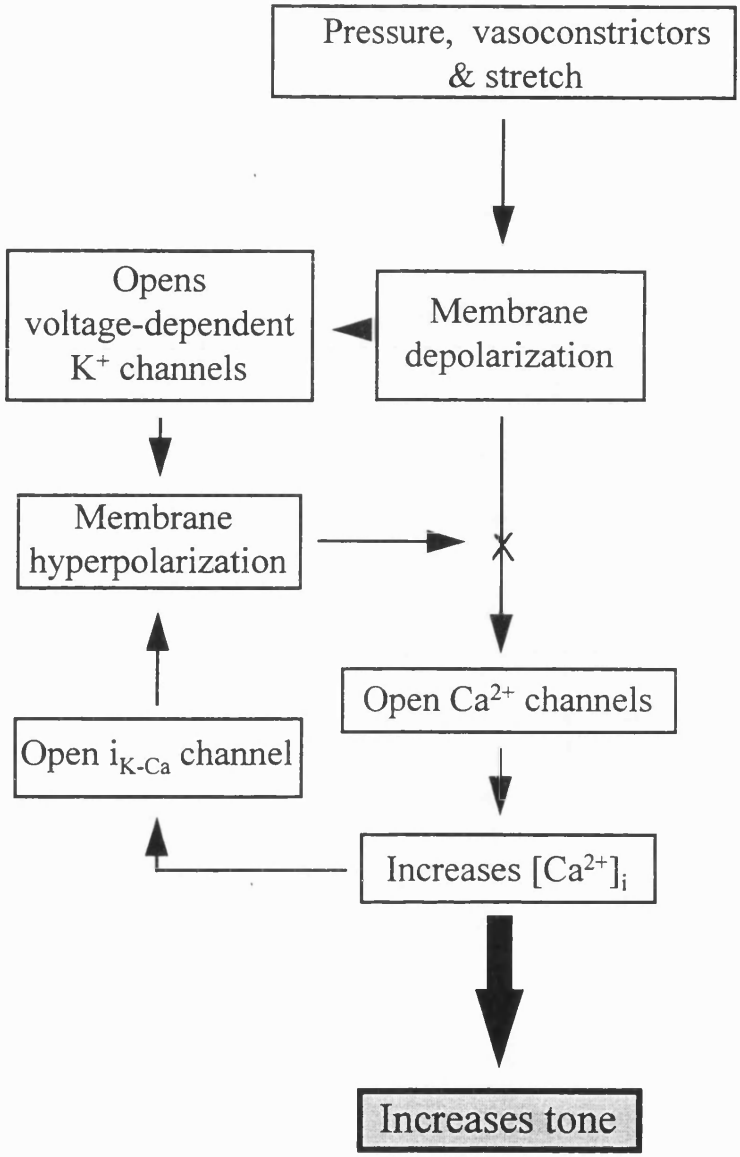


Figure I6: Flow diagram representing the roles of voltage operated and Ca^{2+}-sensitive K^+ channels in vascular smooth muscle contraction. Stretching of the vessel wall leads to membrane depolarization. This leads to opening of voltage-dependent K^+ channels and voltage-sensitive Ca^{2+} channels. This increase in intracellular Ca^{2+} levels also opens Ca^{2+}-sensitive K^+ channels.

1.11.2 K^+ Channels

The field of K^+ channel research is rapidly expanding. To date, there have been 4 major channels studied in vascular smooth muscle (Daut *et al.,* 1994; Nelson *et al.,* 1995).

1.11.3 Ca^{2+}-Activated K^+ Channels

This class of channel can be further divided on the basis of single channel conductance. As such, large conductance (BK; 200pS) and small conductance (SK; 6-22pS) channels have been distinguished. The putative physiological role of the Ca^{2+}-activated K^+ channel is revealed by its dependency on intracellular Ca^{2+}, the probability of the channel opening increases as intracellular Ca^{2+} increases. In addition, the open probability also increases as the membrane depolarizes.

Ca^{2+}-activated K^+ channels are blocked by low concentrations of tetraethylammonium (<1mM) and iberotoxin, while small conductance channels, but not large conductance channels, are inhibited by apamin. Large conductance channels are inhibited by charybdotoxin and penitrem A. Apamin has been demonstrated to contract porcine coronary artery ring preparations, demonstrating these channels maybe open under resting conditions (O'Rourke 1996). These channels may have a role in the termination of contraction due to myogenic tone. This term describes a process where an increase in transmural pressure in a vessel, leads to depolarization and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. This elevation in cytoplasmic Ca^{2+} will stimulate these channels and oppose the depolarization which will, in turn, lead to activation of L-type Ca^{2+} channels (see figure I6).

1.11.4 ATP-Sensitive K^+ Channel

This large class of channels is regulated by purine derivatives of cellular metabolism with intracellular ATP closing and intracellular ADP opening the channel (Zhang & Bolton 1995). The nucleotide di- and triphosphates are thought to bind to distinct regions on the channel complex. In addition, these channels have also been demonstrated to be activated by adenosine and acetylcholine through a G-protein linked mechanism and inhibited by intracellular magnesium (Terzic *et al.,* 1995).

Under conditions where $[ATP]_i$ is low and $[ADP]_i$ is high, *e.g.* metabolic stress and hypoxia, these channels open, K^+ flows down its concentration gradient, hyperpolarizing the membrane and thus stabilizing the cell. This means that the channel has a key role in the metabolic regulation of blood flow. In the coronary circulation, there appears to be species variation in the role of these channels. A study in the isolated rat heart demonstrated that the ATP-sensitive channel inhibitor glibenclamide caused a reduction in coronary flow and left ventricular performance (Randall 1995). In porcine coronary artery ring preparations, however, glibenclamide was shown to have no effect on tension (O'Rourke 1996).

Differences between tissues are also highly dependent on their oxygenation state and levels of intracellular ATP and ADP. Their pivotal role in metabolically active cells makes ATP-sensitive K^+ channels susceptible targets for a variety of endogenous vasodilators *e.g.* nitric oxide (NO) and adenosine (Samaha *et al.,* 1992; Quayle & Standen 1994).

1.11.5. Delayed Rectifier K^+ Channels

Delayed rectifier K^+ channels are voltage dependent, being activated by membrane depolarization (as the membrane depolarizes, K^+ efflux rises sharply) and are insensitive to intracellular Ca^{2+} and ATP. These channels are inhibited by 4-aminopyridine and tetraethylammonium (>10mM). Glibenclamide will inhibit these channels, but the concentrations required are relatively high compared to those selective for ATP-sensitive channels (>100μM).

Since these channels are activated by membrane depolarization, they play an important role in repolarization in electrically excitable vascular smooth muscle cells. In cells which do not fire action potentials, these channels may play a role in limiting membrane depolarization in response to pressure or vasoconstrictive agents.

Studies of these channels measured membrane potential and diameter of the vessels in pressurized small (100-200μM) cerebral arteries from rabbits. It was demonstrated that increases in transmural pressure from 20 to 60 and 80 mmHg led to a decrease in vessel diameter and membrane potential (from -62±5 to -51±4 and -40±2mV) (Robertson *et al.,* 1994). These constrictions were attenuated by removal of external Ca^{2+} or di-hydropyridines and augmented by 4-aminopyridine (Knot *et al.,* 1995). 4-AP also causes a contractile response in porcine coronary artery rings indicating that delayed-rectifier K^+ channels are present in these vessels (O'Rourke 1996).

1.11.6 Inward Rectifier K^+ Channel

When the three channels listed above are excited by the appropriate stimuli, K^+ leaves the cell. There is, however, a channel in which K^+ will enter the cell more readily as opposed to leave. This class of channel is known as inward rectifier. These channels are activated by hyperpolarization, with currents being smaller compared to other K^+ channels. They are sensitive to barium and caesium but glibenclamide and 4-aminopyridine have little effect. These channels tend to stabilize membrane potential at negative values. Their presence in coronary and cerebral arterioles is thought to underlie the changes in vessel tone associated with alterations in extracellular K^+ concentration (Knot *et al.,* 1996). Decreases in the extracellular K^+ concentration causes a fall in conductance and depolarization. So, although the K^+ equilibrium becomes more negative, reducing the number of open inward rectifiers will cause depolarization and contraction.

Type of K^+ channel	Physiological function ?	Pharmacological antagonist
Ca^{2+}-activated	Dynamic membrane potential control in response to increase in $[Ca^{2+}]_i$	Tetraethylammonium (<1mM) Animal toxins (iberiotoxin, apamin, charybdotoxin)
ATP-sensitive	Membrane potential control in response to metabolic changes	Sulphonylureas (Glibenclamide)
Delayed Rectifier	Membrane potential control in response to changes in depolarization	4-Aminopyridine
Inward Rectifier	Membrane potential control in response to changes in $[K^+]_o$	Low concentrations of Ba^{2+}

Table I2: Summary of K^+ channel properties in vascular smooth muscle.

1.12 Coronary Circulation

The blood flow to the myocardium occurs via the coronary circulation. The coronary arteries supply blood to a tissue that is continuously active and cannot sustain an oxygen debt. The circulation consists of two coronary arteries, the left (which divides to form two branches; the left anterior descending and circumflex arteries) and the right. These main coronary arteries are located on the surface of the heart with further branches penetrating into the myocardium. These vessels are responsible for meeting the metabolic demands of the myocardium with tight regulatory mechanisms including humoral, metabolic and neuronal factors.

Venous effluent from the myocardium is drained by the coronary veins. Nearly all the blood from the left coronary artery, about 75% of coronary blood flow, leaves via the coronary sinus. Blood leaving the right ventricle will flow directly into the right atrium via the anterior cardiac veins. Some of the blood exiting left coronary circulation also leaves via the Thebesian vessels, which drain into the left ventricle.

The dependence of myocardial cells on aerobic metabolism suggests that the coronary circulation has well-developed means of regulating and adjusting coronary blood flow to meet the demands of oxygen requirement of the myocardium. One substance that is integral to the metabolic regulation of coronary blood flow is adenosine. It is released during myocardial ischaemia and dilates small diameter coronary vessels in order to increase flow (Rubio & Berne 1969; Stepp *et al.* 1996).

The myocardium not only exerts a chemical influence on coronary tone, but a physical one. The majority of coronary flow occurs when the cycle is in diastole.

1.13 pH And Its Affects On Smooth Muscle

Smooth muscle cells perform many functions essential for the normal functioning of the human body, such as control of blood vessels (and hence blood pressure), digestion, micturation and respiration. The powerful relationship (Pouseilles Law) between flow of blood and the radius of a vessel (flow $\propto$ radius^4) is such that doubling the vessel radius will increase flow 16-fold,. Bearing this in mind, it is obvious that any manoeuvre which affects vessel tone can have huge effects on blood flow. The effects of pH on vascular smooth muscle vary depending on the tissue studied (Aalkjaer & Poston 1996). There are also differences within the same tissue type *i.e.* rabbit coronary artery may respond differently compared to rat coronary artery..

1.13.1 Blood Vessel Reactivity

1.13.1.1 Non-Coronary Vessels

Perhaps the most commonly utilised experimental tissue for the investigation concerning the interactions of pH and vascular smooth muscle tone is the rat mesenteric circulation.

Changes in extracellular pH alter intracellular pH in the same direction of the pH change. Therefore, increased acidity of the extracellular solution will acidify the cytoplasm. This has been demonstrated extensively in the mesenteric circulation of the rat (Austin *et al.,* 1993 a & b). These studies used strips of rat mesenteric resistance vessels using the pH-sensitive flurophore SNARF-1 to measure intracellular pH in HEPES and HCO_3^--CO_2 buffered solutions. Extracellular acidosis (pH 7.4 to 6.9) altered intracellular pH (pH 7.3 to 6.95) with the time to half-peak response of approximately 40 seconds and an intracellular : extracellular pH ratio of 0.73 unit. This acidification of the cytoplasm was associated with a decrease in tension. In contrast, extracellular alkalosis (pH 7.4 to 7.9) was associated with intracellular alkalosis (pH 7.3 to 7.7) and an increase in tension. Similar findings were obtained using isolated rat cerebral arteries (Tian *et al.,* 1995).

Further studies performed in isolated rat portal vein (Taggart *et al.,* 1994) have used weak acids and bases to alter intracellular pH while keeping extracellular pH constant. In these experiments, the acid (20mM sodium butyrate) and base (20mM trimethylamine) caused equivalent pH and tension changes to those seen in the mesenteric vessel. As such, butyrate induced intracellular acidosis and inhibited the spontaneous activity while alkalosis augmented the spontaneous activity. Furthermore, in preparations that were not constricted, butyrate (40mM) caused intracellular acidification but no change in tension, while KCl-contracted tissue relaxed in response to a similar treatment (Austin & Wray 1994).

In contrast, alkalization induced by 20mM NH_4Cl in a HEPES-buffered rat portal vein preparation decreased intracellular Ca^{2+} levels and reversibly inhibited contractions induced by 60mM K^+. An intracellular acidosis produced by propionate caused the opposite effects, namely an increase in intracellular Ca^{2+} levels and potentiated the K^+ contractions (Iino *et al.,* 1994).

The technique of using an ammonium salt pre-pulse to alter intracellular pH without alteration of extracellular pH has been investigated (Furtado 1987). Application of 30mM NH_4Cl to isometrically mounted rabbit aortic rings bathed in HCO_3^--buffered Krebs-Henseleit solution caused a transient relaxation in rings pre-contracted with noradrenaline. On wash-out of the NH_4Cl, a transient constriction was observed which returned to control level.

In an extensive study performed on isolated rings of canine left anterior descending coronary artery and mesenteric artery mounted for isometric contraction in HCO_3^--CO_2-buffered Krebs-Henseleit solution, pre-constricted with prostaglandin $F_{2\alpha}$, coronary artery rings relaxed in a concentration-dependent manner in response to the application of ammonium salts (Feletou *et al.,* 1989).

Interestingly, this study also demonstrated that the constrictor agent used can affect the results. Constriction with 40mM KCl, but not 30mM KCl, resulted in loss of the dilation in response to the alkalosis produced by the application of the ammonium salts. This was independent of the degree of constriction obtained. Tension recovered in the presence of the ammonium ions. On wash-out of the NH_4Cl, a transient constriction occurred (Feletou *et al.,* 1989) mesenteric vessels showed similar responses to the

coronary artery rings. It was also demonstrated that the relaxation in the mesenteric preparation was independent of changes in membrane potential and guanylate cyclase activity. This would indicate that the relaxation was not due to EDHF or NO.

Further differences between wire-mounted and cannulated preparations with respect to intracellular pH, Ca^{2+} and contraction were highlighted in a study using rabbit mesenteric artery and HEPES-buffered solutions (Nakanishi *et al.,* 1996). Data from these experiments showed that, depending on the manner in which the mesenteric vessel was prepared (wire mounted or cannulated), and the contractile agent used (noradrenaline or increased extracellular K^+ levels), different results were obtained when acidosis was induced with increased pCO_2.

The study by Nakanishi *et al.,* (1996) measured tension, intracellular pH and intracellular calcium levels. Not surprisingly, the authors concluded that the effects of acidosis on vascular tone is dependent on the contractile agent and type of preparation studied. One further proposition was that intracellular Ca^{2+} may partly regulate acidosis-induced responses. This study also demonstrated that the relaxations were independent of endothelial removal, NO, prostaglandin or α and β adrenoceptors.

These studies all indicate that alkalosis induced by the application of NH_4Cl is associated with relaxation of the respective tissues.

However, changes in tension associated with NH_4Cl application (alkalosis) have been shown to be sensitive to the voltage-sensitive channel blocker nifedipine in rat portal vein (Taggart *et al.,* 1995) and porcine coronary artery (Wakabayashi *et al.,* 1996). In contrast to this, extracellular acidosis has been demonstrated to inhibit current flowing through L-type Ca^{2+} channels in guinea-pig basilar artery (West *et al.,* 1992), bovine pial and porcine coronary arteries (Klöckner *et al.,* 1994a), rat portal vein (Iino *et al.,* 1994) and porcine tracheal smooth muscle (Yamakage *et al.,* 1995).

This acidosis-induced constriction has also been demonstrated in α-toxin-permeabilized isolated rat uterine muscle (Crichton *et al.,* 1993). Uterine muscle strips were bathed in HEPES buffered solution (pH 7.2) and the Ca^{2+} indicator INDO-1 was used to measure intracellular Ca^{2+}. Extracellular acidosis (pH 7.2 to 6.7) elicited an increase in tension without a change in Ca^{2+} levels. Normalization of tension-Ca^{2+} responses at the two pH values showed a sensitization of the contractile apparatus at intracellular Ca^{2+} levels above 1μM. Similar sensitization has been illustrated in canine tracheal smooth muscle strips (Yamakage *et al.,* 1995). Alkalinization (pH 7.8; achieved by addition of NaOH) significantly increased intracellular Ca^{2+} levels and enhanced muscle contraction in response to K^+ depolarization but did not alter the Ca^{2+}-tension relationship. In contrast to this, acidosis (pH 7.0; achieved by addition of HCl) decreased intracellular Ca^{2+} levels without changing the Ca^{2+}-tension relationship. The authors suggest that this the decrease in intracellular Ca^{2+} without changes in tension represents sensitization induced by acidosis.

Therefore, the responses observed in acidotic conditions can not be correlated to Ca^{2+} levels implicating alterations in the sensitivity of the contractile machinery.

1.13.1.2 Coronary Vessels

In a study using isolated guinea-pig hearts perfused with HCO_3^--buffered solution (basal flow rate 9.4ml.minute^{-1}, cardiac contractility measured via a thread attached to the apex of the heart), it was demonstrated that heart rates, cardiac contraction and coronary flow rates were affected by alterations in extracellular pH. In addition to a negative chronotropic and inotropic effect, coronary flow rate increased in response to acidosis (extracellular pH 7.48 to 7.03) (McElroy *et al.,* 1958). When the opposite changes in pH were made (increased HCO_3^- and decreased CO_2), heart rate and cardiac contraction increased while coronary flow rate decreased. While alterations in the HCO_3^- concentration or CO_2 level were made that did not result in alterations in pH, no change in the experimental parameters were observed.

As mentioned earlier, accumulation of CO_2 occurs during ischaemia. In one study performed on open-chested dogs, coronary blood flow was supplied to the cannulated left main coronary artery from the femoral artery. Changes in pCO_2 were made locally in the circulatory system, with each alteration of pCO_2 being made at a constant flow rate (Case *et al.,* 1976). Progressive increases in coronary sinus pCO_2 lead to a decrease in coronary vascular resistance (vasodilation).

Early work by Wang and Katz (1965) also utilised a canine preparation in which coronary flow was controlled by a double-barrelled catheter passed retrogradely through the descending thoracic aorta. Infusions of the weak acid 5,5-dimethyl-2, 4-oxazolidinedion (DMO) into the coronary arteries caused a decrease in cardiac contractility (measured with a strain gauge attached to the right ventricle) and heart rate.

Interestingly, the authors noted that coronary flow displayed a bi-phasic response, initially increasing before decreasing. They postulated that the decrease in flow (vasoconstriction) was invariably associated with the decrease in cardiac contractility, possibly through a reduction in the amount of a metabolic vasodilator *e.g.* adenosine.

Contrary to this suggestion, acidosis induced by increasing pCO_2 (pH 7.1 and 6.8) was demonstrated to increase adenosine release in isolated rabbit hearts (Mustafa *et al.,* 1984). However, a recent study in rat hearts showed acidosis to decrease adenosine release (Ochi *et al.,* 1991).

In a metabolic study using electrically paced (300 beats $minute^{-1}$) perfused (constant head of pressure 85cm above heart) perfused rat heart, Schaffer *et al.* (1978) described acidotic-induced vasoconstriction. Respiratory acidosis (pH 6.2) induced a reduction in coronary flow to 20% of control values. Interestingly, alkalosis (pH 7.8, :unspecified method) caused no significant affect on coronary flow (Schaffer *et al.,* 1978; Watters *et al.* 1987).

Another study using porcine coronary vascular smooth muscle has shown that the acidosis-induced dilation can be attenuated by the ATP-sensitive K^+ channel blocker glibenclamide (Ishizaka *et al.,* 1996). The dilation was greater in preparations constricted with 1μM U46619 than with 29mM KCl, even though the effect of the constrictor was not as great.

1.14 Intracellular Ca^{2+} And pH

The interactions between intracellular pH and Ca^{2+} concentration are complex and are dependent on many factors (Daugirdas *et al.,* 1992). Alkalinization produced by NH_4Cl caused intracellular Ca^{2+} to increase in a vascular smooth muscle cell line A7r5. This increase was not dependent on external Ca^{2+}, but could be inhibited by TMB-8, a compound which blocks Ca^{2+} uptake and release from the sarcoplasmic reticulum. This result suggests that there are two separate pools of Ca^{2+} (Daugirdas *et al.,* 1992).

Acidosis has also been shown to increase intracellular Ca^{2+} in rabbit portal vein cells (Iino *et al.,* 1994). In this study, contractions to 60mM KCl were recorded from rings of portal veins and intracellular Ca^{2+} and pH were recorded using FURA-2AM and 4', 5' - dimethyl-5,6-carboxyl-fluorscein (DMCF). Caution must be used when considering the conclusion drawn from this study since it was performed using HEPES-buffered experimental solutions, and it has been demonstrated that pH changes induced by various agonists can be attenuated in HEPES-buffered solutions (Douglas *et al.,* 1991; Zierath *et al.,* 1992). Under the experimental conditions used, intracellular acidosis (20mM propionate) decreased Ca^{2+} current but it potentiated KCl-induced contraction and increased intracellular Ca^{2+} concentration (Iino *et al.,* 1994).

A recent study performed using small strips of rat mesenteric vessel simultaneously measured tension, intracellular pH (carboxy-SNARF) and Ca^{2+} (INDO-1) whilst examining the effects of extracellular pH (Austin *et al.,* 1996). Increases in extracellular pH (alkalinization) increased tension, intracellular pH and intracellular Ca^{2+}. Acidosis

had the opposite effect. Interestingly, the changes in intracellular Ca^{2+} were always preceded by changes in intracellular pH, irrespective of which direction the change took.

Studies have demonstrated that the technique employed has implications for the study of small arteries *in vitro*. For example, mesenteric arteries have been compared in a pressurized flow chamber with a wire myograph at transmural pressures of 44mmHg (Falloon *et al.,* 1995). In this study it was found that endothelium-intact pressurized preparations were significantly more sensitive to noadrenaline and to angiotensin II, while wire mounted preparations were less responsive. Differences in noradrenaline were shown to be due to the enhanced influence of neuronal amine uptake in wire-mounted preparations. Similar differences were observed in a separate study performed on similar preparations (Buus *et al.,* 1994; Nakanishi *et al.* 1996).

1.15 Aims

In view of the paucity of studies performed in the intact coronary circulation of the rat, a species which is widely used for the study of ischaemia and reperfusion, experiments have been performed to characterise the response of the tissue to acidosis:

1. To investigate the effects of metabolic acidosis (pH 7.2-6.0), applied at a constant flow rate, or constant head of pressure, on the intact coronary circulation of the isolated perfused rat heart.

2. To compare the effects of different buffer systems on basal and acidosis-induced alterations in the measured experimental parameters.

3. To further characterise the modifications in experimental parameters induced by acidosis.

4. To determine whether the acidosis-induced changes are species specific and particular to the vascular tissue used.

2 METHODS

2.1 Isolated Heart Preparation

Male Wistar rats (300-330g) or Dunkin-Hartley guinea pigs (300-320g) were anaesthetised with sodium pentobarbitone (120 $mg.kg^{-1}$ *i.p.*.) prior to cervical dislocation. The heart was excised then perfused, via its own circulation, at a rate of 10 $ml.minute^{-1}$ with oxygenated Krebs-Henseleit solution (composition see Table M1) at 37°C & pH 7.4 using a modified Langendorff technique. Thebesian vein effluent was removed with a needle inserted into the left ventricle. Since the system utilises a constant flow rate, any change in coronary tone is registered as a change in coronary perfusion pressure (mmHg) measured by a pressure transducer attached to a side arm on the aortic cannula. Developed tension (grams) was measured, under a resting tension of 2 grams, via an isometric tension transducer attached to the apex of the heart. This signal was also used to monitor heart rate (beats $minute^{-1}$).

2.2 Isolated Rat Superior Mesenteric Bed

Following ligation of the colonic artery and careful dissection clear of the gastrointestinal tract, the superior mesenteric bed was perfused via the superior mesenteric artery at a rate of 5 $ml.minute^{-1}$. Mesenteric perfusion pressure (mmHg) was measured with a pressure transducer, similar to the method used to obtain coronary perfusion pressure.

2.3 Isolated Porcine Coronary Artery Ring Preparation

Pig hearts were obtained from a local abattoir within an hour of death. Left coronary arteries were carefully removed and cleaned of any connective tissue. Rings were mounted isometrically in an organ bath with Krebs-Henseleit solution (37°C) and allowed to equilibrate for 30 minutes at a resting tension of 1g.

2.4 Isolated Rat Aorta Ring Preparation

Following pentobarbitone anaesthesia and cervical dislocation, the thoracic region of the aorta was carefully removed and placed in oxygenated Krebs-Henseleit solution of an identical composition to the solution employed in isolated heart experiments. After removing the initial 5 mm, rings of length 2-3mm were mounted isometrically (resting tension 1g) in an organ bath containing oxygenated Krebs-Henseleit solution (37°C).

2.5 $[Ca^{2+}]$ Determination

$[Ca^{2+}]$ was determined using a Russell pH Limited (model 93-3209) and a Corning pH/mV meter 140. A calibration curve was constructed for $[Ca^{2+}]$ between 10^{-4} to 10^{-2}M. When needed, equilibrated Krebs-Henseleit solution (pH 7.40 and pH 6.8) were read and their $[Ca^{2+}]$ obtained from the calibration curve.

Table M1: Composition of experimental buffers (mM).

	Krebs-Henseleit Solution (pH7.4)	Krebs-Henseleit Solution (pH6.8)	HEPES-buffered Tyrode (pH7.4)
NaCl	118	118	144
$NaHCO_3$	25	7*	-
D-Glucose	11.6	11.6	11.6
KCl	4.7	4.7	5.9
KH_2PO_4	1.2	1.2	-
$MgSO_4$	1.2	1.2	1.2
HEPES	-	-	6
$CaCl_2$	1.23	1.23	1.23
pH	7.4	6.8	7.4$
GAS	95%O_2/5%CO_2	95%O2/5%CO_2	AIR

* The figure stated is the concentration required to achieve pH 6.8. Concentration required for other pH levels used appear in results section.

$ HEPES-buffered Tyrode solution pH was altered using 1M NaOH

2.6 Experimental Protocols

2.6.1 Experimental Solutions

In order to investigate the effects of acidosis on the tissues, it was necessary to maintain stable perfusate pH levels (pH 7.4-6.2). To achieve this, a HCO_3^--free Krebs-Henseleit solution was equilibrated with 95%O_2:5%CO_2 gas mixture for 30 minutes, constantly measuring pH. Once stable, HCO_3^- was then added to achieve the required pH.

In experiments performed using hypercarbic respiratory acidosis, HCO_3^--containing Krebs-Henseleit solution was again equilibrated with 95%O_2:5%CO_2 gas mixture. Additional CO_2 was passed through the perfusate. To calculate the partial pressure of CO_2 (pCO_2 measured as mmHg) necessary to achieve acid pH, a derivation of Henderson-Hasselbach equation was used:

$$pH = 6.1 + \log [HCO_3^-]/[CO_2]$$

2.6.2 Isolated Heart Preparation

2.6.2.1 Control Responses

Following a 15 minute equilibration period at pH 7.4 metabolic acidosis (7.4-6.0) was applied for a duration of 5 minutes and its effects on heart rate (HR beats minute^{-1}), developed tension (DT grams) and coronary perfusion pressure (CPP mmHg) measured. This was followed by a 10 minute recovery period at pH 7.4.

This manoeuvre was repeated 4 times to ensure that there was no difference between the acidotic challenges.

Results from the experiments described on the previous page revealed the acidosis-induced alterations in the experimental parameters to be pH-dependent. Extensive studies were then performed to further characterise the effects induced by metabolic acidosis (pH 6.8) since this degree of acidosis produced sub-maximal increases in CPP.

2.6.2.2 Studies Using Other Buffer Systems

These experiments were performed in both rat and guinea pig hearts.

Following the control responses to metabolic acidosis (pH 6.8) described above and the wash-out period, either respiratory acidosis (pH 7.4) or HEPES-buffered Tyrode (pH 7.4) was applied for 10 minutes. When parameters stabilised, either respiratory acidosis

of HEPES buffered Tyrode (both pH 6.8) were applied for 5 minutes and their effects on the parameters noted, followed by a 10 minute washout period.

In experiments investigating the different buffer systems used, the first series of responses to metabolic acidosis were used as control.

2.6.2.3 Constant Head Of Pressure Experiments

Isolated rat hearts were perfused as stated above. Following a control response to metabolic acidosis (pH 6.8), perfusion was switched from constant flow rate ($10ml.minute^{-1}$) to a constant head of pressure (100cm above aortic cannula). Following equilibration, metabolic acidosis was applied in a similar regime as above.

Calculations of coronary resistance were performed following the equations as utilised by Ferrera *et al* (1995).:

Coronary resistance = coronary perfusion pressure.wet weight of heart/coronary flow

It is assumed that heart weight did not increase and since coronary flow was constant (except in constant head of pressure experiments), coronary resistance reflects coronary perfusion pressure

2.7 Characterization Of Acidosis-Induced Responses

An extensive study was performed to further investigate possible mechanism of action of the observed changes. These were all carried out using metabolic acidosis (pH 6.8).

2.7.1 Manipulation Of Cardiac Contraction

As acidosis depresses cardiac contraction, some experiments were carried out to investigate whether the changes in CPP were secondary to the depression of cardiac contractility. In order to ascertain whether the effects of metabolic acidosis (pH 6.8) on the vascular smooth muscle of the coronary circulation occurred as a direct effect. two methods were employed.

Control responses to metabolic acidosis (pH 6.8) were obtained as previously stated. Then, perfusate potassium concentration was increased from 5.9 to 15mM in order to arrest cardiac contraction. Following a 5 minute equilibration period, metabolic acidosis (pH 6.8) was re-applied for five minutes followed by a10 minute recovery period. This was repeated three times before the potassium concentration was returned to 5.9mM and metabolic acidosis (pH 6.8) was re-applied.

In a further set of experiments following control responses, hearts were electrically paced via electrodes on the aortic cannula and the tip of the right ventricle (stimulation parameters: duration 5msecs; strength 7V). The rate was raised in increments of 2Hz.. from 6 to 16Hz. and its effect on cardiac contraction and coronary perfusion pressure

noted. When these parameters had stabilised, metabolic acidosis (pH 6.8) was re-applied for 5 minutes. Following the 10 minute recovery period at pH 7.4, pacing was stopped and the heart allowed to regain its normal rate before metabolic acidosis (pH 6.8) was applied again.

Results obtained with high K^+ and pacing were compared to their respective control values for statistical analysis.

2.7.2 Effect Of 8-PT, Indomethacin, NO-ARG, Phentolamine And ZD 1542 On Acidosis-Induced Responses

The observed effects of metabolic acidosis on CPP could be due to either an enhancement/attenuation of an endogenous vasoconstrictor or vasodilator substance respectively. Some antagonists were applied in order to investigate any possible roles for several substances

Following control responses to metabolic acidosis (pH 6.8) in isolated rat hearts, 8-PT (10μM), indomethacin (10μM), N-NITRO-L-ARGININE (NO-ARG 100μM), phentolamine (10μM) or the dual TxA_2 receptor antagonist and synthase inhibitor ZD 1542 (0.1 10μM) were applied for 15 minutes and their effects on basal parameters observed. Acidosis was re-applied and responses compared to their respective controls.

2.7.3 *Investigation Into The Role Of Extracellular Ca^{2+}*

The role of extracellular Ca^{2+} in the acidosis-induced coronary vasoconstriction was evaluated using several methods.

Firstly, following control responses to metabolic acidosis (pH 6.8), Ca^{2+} was removed from the perfusate for 5 minutes and the effects noted. Metabolic acidosis was then induced again in the Ca^{2+}-free condition and results compared to control.

Secondly, several pharmacological blockers of L-type Ca^{2+} channels were employed. Again, following control responses to metabolic acidosis (pH 6.8) amlodipine (100nM), nifedipine (1-100nM), verapamil (1μM) were added for 5 minutes and their effects on cardiac contraction and vascular smooth muscle tone noted. Acidosis was then applied in the presence of these Ca^{2+} channel antagonists.

Further experiments were performed in some nifedipine studies to test the viability of the coronary vascular smooth muscle. Control constrictions to a bolus dose of 30pmoles endothelin-1 were compared to constrictions to the same bolus of endothelin-1 in the presence of 100nM nifedipine.

2.7.4 *Other Vasodilators*

Since experiments performed with the Ca^{2+} antagonists demonstrated that this class of compound caused vasodilation, further experiments were performed with other vasodilatory procedures to control for this. In these studies coronary vasodilation was

produced by hypoxic perfusate (95%N_2:5%CO_2) and 100nM P1Pi, the isopropyl ester of palmitoyl carnitine (Reeves *et al.*1995). These were applied and their effects on responses to acidosis noted.

2.7.5 *Alterations Of Intracellular pH Only*

Applications of NH_4Cl and weak acids can be extremely useful experimentally, as they can cause an intracellular acidosis whilst maintaining extracellular pH constant. As described in the introduction, application of NH_4Cl leads to intracellular alkalinization while removal causes intracellular acidosis, while butyric acid (10mM) acts directly to decrease intracellular pH. These compounds were applied separately for a 5 minute period to investigate the effects of intracellular acidosis on the isolated rat heart.

2.7.6 *K^+ Channel Blocker Studies*

K^+ channels play a pivotal role in the determination of the membrane potential of vascular smooth muscle cells. Since voltage-sensitive Ca^{2+} channels are activated by membrane depolarization, K^+ channels are central in this phenomenon. To assess their role in the coronary circulation of the rat, a variety of K^+ channel blockers (4-AP, TBA and TEA 100nM-10mM; glib 0.1nM-10μM; pen A 0.1nM-100nM) were perfused through rat hearts for 5 minutes followed by a 10 minute recovery period.

In a separate series of experiments, coronary contractions induced by K^+ channel blockers were tested for their sensitivity to nifedipine (100nM).

2.8 Other Vascular Preparations

Other vascular preparations were used to investigate whether the observed changes in response to acidosis were specific to the coronary circulation of the rat.

2.8.1 Isolated Superior Mesenteric Vascular Bed

A similar protocol to that used in the heart was used to examine the effects of metabolic or respiratory acidosis (pH 6.8), NH_4Cl (10mM) and butyric acid (10mM) on mesenteric perfusion pressure (MPP mmHg) in a basal state, or pre-constricted with phenylephrine (10μM) or potassium (70mM).

2.8.2 Isometrically Mounted Rat Aorta And Porcine Coronary Artery Ring

In experiments using rat aorta and porcine coronary artery rings the preparations were allowed to equilibrate for thirty minutes under a resting tension of 1g. The effects of acidosis were then examined under basal conditions or when preparations had been pre-constricted with K^+ (42mM) or phenylephrine (>10μM).

A recent study (Furukawa *et al* 1996) described acidotic-induced constriction of rat aortic strips which was sensitive to Ca^{2+} channel antagonists and attenuated under Ca^{2+}-free conditions. In an attempt to replicate this study, experiments using isolated rat aortic rings were performed. After equilibration, high K^+ solution (total $[K^+]$ 42mM)

was added to ascertain preparation viability. After the third such challenge, metabolic acidosis was applied and its effect on tension noted.

In studies using porcine coronary artery rings, preparation viability was tested by a 5 minute application of a solution containing 42mM K^+. Responses to phenylephrine (>10μM) were also recorded. Metabolic acidosis was then applied for 5 minutes and effects on basal tension noted. High K^+ solution was re-applied and effects noted on the potassium-induced constriction.

2.9 Statistical Analysis

Statistical analysis was applied to the experimental data using the Minitab computer program.

2.9.1 The Null Hypothesis

With regard to comparison of data, the null hypothesis (the probability of any significant difference being due to chance alone) was accepted at $P > 0.05$. The null hypothesis was rejected where $P < 0.05$.

2.9.2 Statistical Analyses

2.9.2.1 Parametric Tests

2.9.2.2 Student's t-test

This test was used to compare means of data from samples of control responses versus treatment data, where only two means where compared. The student's t-test was used to compare data from basal levels and acidotic responses.

Prior to using this particular test, data was first analysed using the F-test to determine the heterogeneity of variance. Data was assumed to be acceptable for parametric analysis if the F-test revealed normally distributed data.

2.9.2.3 *Analysis of variance*

Where more than two means were to be compared, analysis of variance was used. When any differences were identified, an appropriate test (Dunnetts) was used to compare to control values.

2.10 *Drugs And Chemicals*

D-Glucose, $CaCl_2$, KCl, KH_2PO_4, $MgSO_4$, $NaCO_3$, NaCl and, $NaHCO_3$ were all purchased form BDH chemicals (Poole, Dorset).

All drugs were purchased from Sigma (Poole, Dorset, U.K.) except BAY K 8644 which was purchased from (Bayer AG), endothelin-1 which was purchased from (Novabiochem UK Ltd.) and ZD 1542 which was a gift from Dr. M.G. Wayne (Zeneca, U.K.) and P1Pi which was synthetised by Dr.M. Rad-Nikam (University of Bath).

BAY K 8644 was dissolved in DMSO to a concentration of 10mM. Serial dilutions were then performed using saline (0.9%) to the required concentration.

Endothelin-1 was dissolved in saline at a concentration of 100μM.

Indomethacin was dissolved in minimum volume of Krebs-Henseleit solution containing $NaCO_3$ (1M) prior to final dilution in Krebs-Henseleit solution.

NO-ARG was diluted in a minimum volume of Krebs-Henseleit solution and sonicated to aid the dissolving process. This was then added to the final volume of Krebs-Henseleit solution.

To prevent oxidation, phenylephrine was dissolved in saline containing ascorbic acid (100μM).

3.0 RESULTS

3.1. Experimental Solutions and pH Changes

Initial experiments were performed to establish the dependence of perfusate pH on the concentration of $NaHCO_3$, plus the quantity of CO_2 required to acidify the solutions when needed. The [$NaHCO_3$] required for pH 6.1, 6.4, 6.8, 7.0, 7.2 and 7.4 was 2.2, 3.6, 6.2, 11, 18.6 and 25mM respectively (Figure R1).

By using the modified Henderson-Hasselbach equation stated earlier pCO_2 for normal Krebs-Henseleit solution ([$NaHCO_3$] 25mM; pH 7.4) was calculated to be 41.6mmHg. In order to cause hypercarbic acidosis (pH 6.8) in the solution, pCO_2 was increased to 165.7mmHg.

Reports exist stating that reductions in [$NaHCO_3$] can alter the free Ca^{2+} concentration within the perfusate (Fry & Poole-Wilson 1981). Using a Ca^{2+} electrode it was shown that there was no difference in ionised Ca^{2+} levels recorded between Krebs-Henseleit solution (pH 7.4) and metabolic acidotic solution (pH 6.8).

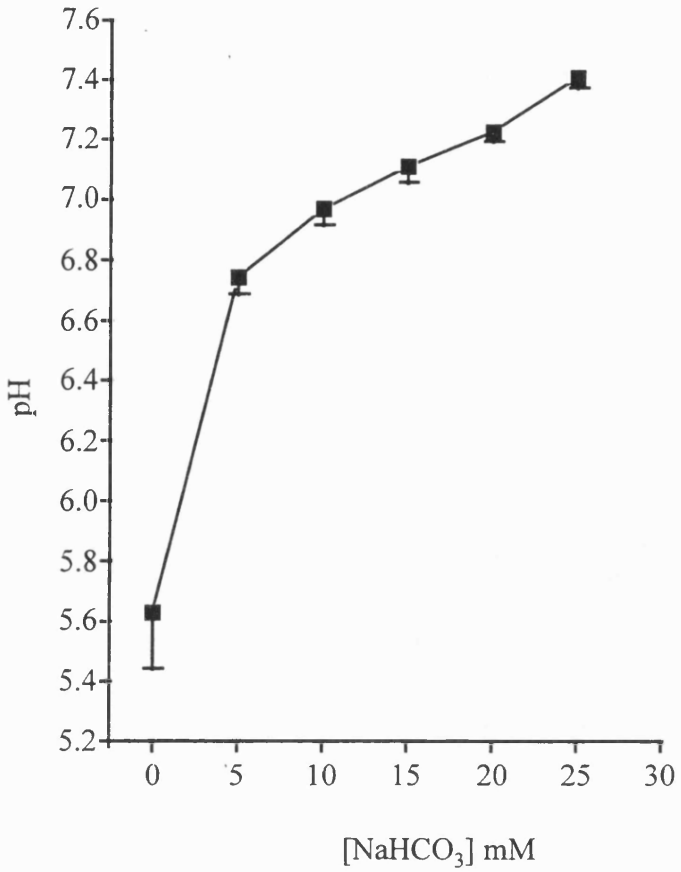


Figure R1: Graph showing relationship between the concentration of $NaHCO_3$ and pH of experimental Krebs-Henseleit solution (mean ± s.e.m., n=5). It can be seen that over the pH range of 6.8-7.4, there was a linear relationship between [$NaHCO_3$] and pH.

3.2. *Effects Of Acidosis On The Isolated Perfused Rat Heart*

3.2.1 *Extracellular pH dependency*

Results from other studies have shown that alterations in extracellular pH are transferred to intracellular pH (Austin & Wray 1993a). So, it must be borne in mind that any effects observed using alterations in extracellular pH might also be due to subsequent alteration in intracellular pH.

All alterations in parameters recorded were dependent on the degree of acidosis applied. As a more severe acidotic challenge was applied (pH 7.2 progressing to pH 6.2), a more pronounced negative effect on heart rate (HR) was noted (HR was not capable of being measured at certain pH levels since DT was depressed to such a degree as to not trigger the rate meter). Figure R2 demonstrates that as the degree of acidosis was increased, by reducing [$NaHCO_3$], coronary constriction increased in a pH-dependent manner.

Figure R3 is a typical experimental trace demonstrating the effects of multiple 5 minute applications of metabolic acidosis (pH 6.8) on coronary perfusion pressure (CPP), developed tension (DT) and heart rate (HR) of the isolated perfused rat heart. It can be seen that there was a transient positive inotropic effect of 2.1±0.3g on perfusion with the acidotic solution, there was also a transient vasodilation of 25±9mmHg which occurred concurrently with this. These changes were followed by a sustained decrease in DT and

a rise in CPP to the values reported in Table R1.

The values quoted in Table R1 are taken at the first of 4 acidotic challenges, at the 5 minute point prior to return to pH 7.4. Acidosis (pH 6.8) caused a negative chronotropic and inotropic effect, plus a maintained coronary constriction. It can also been seen from Table 1 that these changes occurred independently of buffer composition. If acidosis was applied using an increase in pCO_2 or using a HEPES-buffered Tyrode solution, similar trends in the recorded parameters were observed.

Like Krebs-Henseleit solution, all responses were reproducible using the stated methods of producing acidosis (pH 6.8). The only difference was that there was no transient increase in DT or vasodilation when hypercarbic acidosis was applied. All acidosis-induced changes in the isolated rat heart were reversed on return to pH 7.4.

If acidosis (pH 6.8) was applied for a longer period (10 minutes), no further changes occurred in any of the experimental parameters than were observed after 5 minutes.

Distinct changes also occurred when switching between perfusates. Replacing Krebs-Henseleit solution with HEPES-buffered Tyrode solution (both pH 7.4) caused a sustained reduction in DT from 5.1±0.5 to 2.8±1.0 g. This experimental procedure also reduced HR from 313±15 to 180±11 beats $minute^{-1}$ and caused a transient coronary dilation of 20±6mmHg which returned to control levels within 5 minutes. Even though DT and HR were reduced on perfusion with HEPES buffer, further significant reductions were observed when acidosis (pH 6.8) was applied (Table R1).

All of the remaining experiments were done using pH 6.8 since it represents a sub-maximal vasoconstriction in the isolated rat heart.

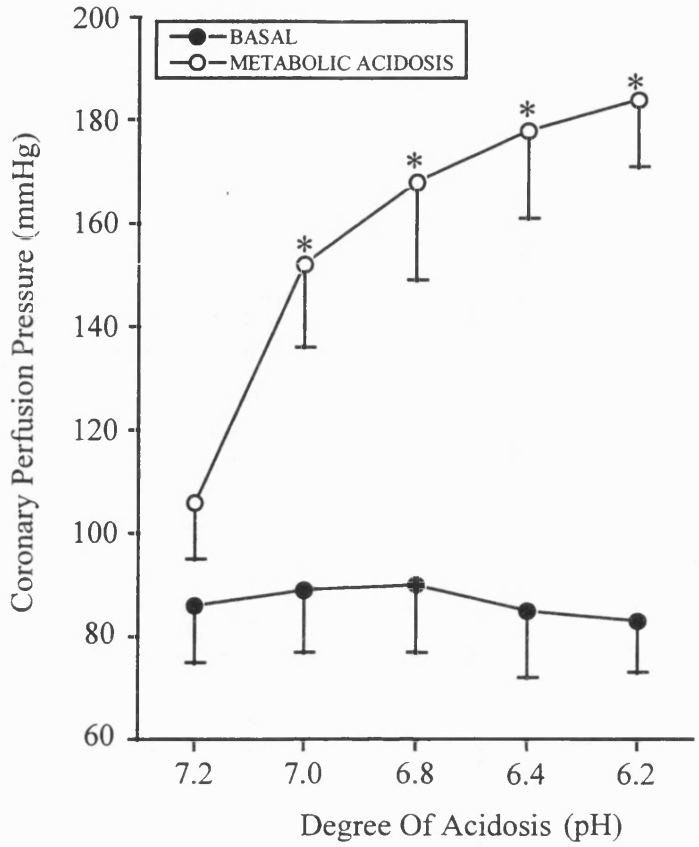


Figure R2: pH response curve showing the effects of graded decreases in pH on the coronary perfusion pressure of the isolated perfused rat heart. It can be seen that as pH becomes more acidic, coronary perfusion pressure increases.

* $p<0.05$ compared to basal levels.

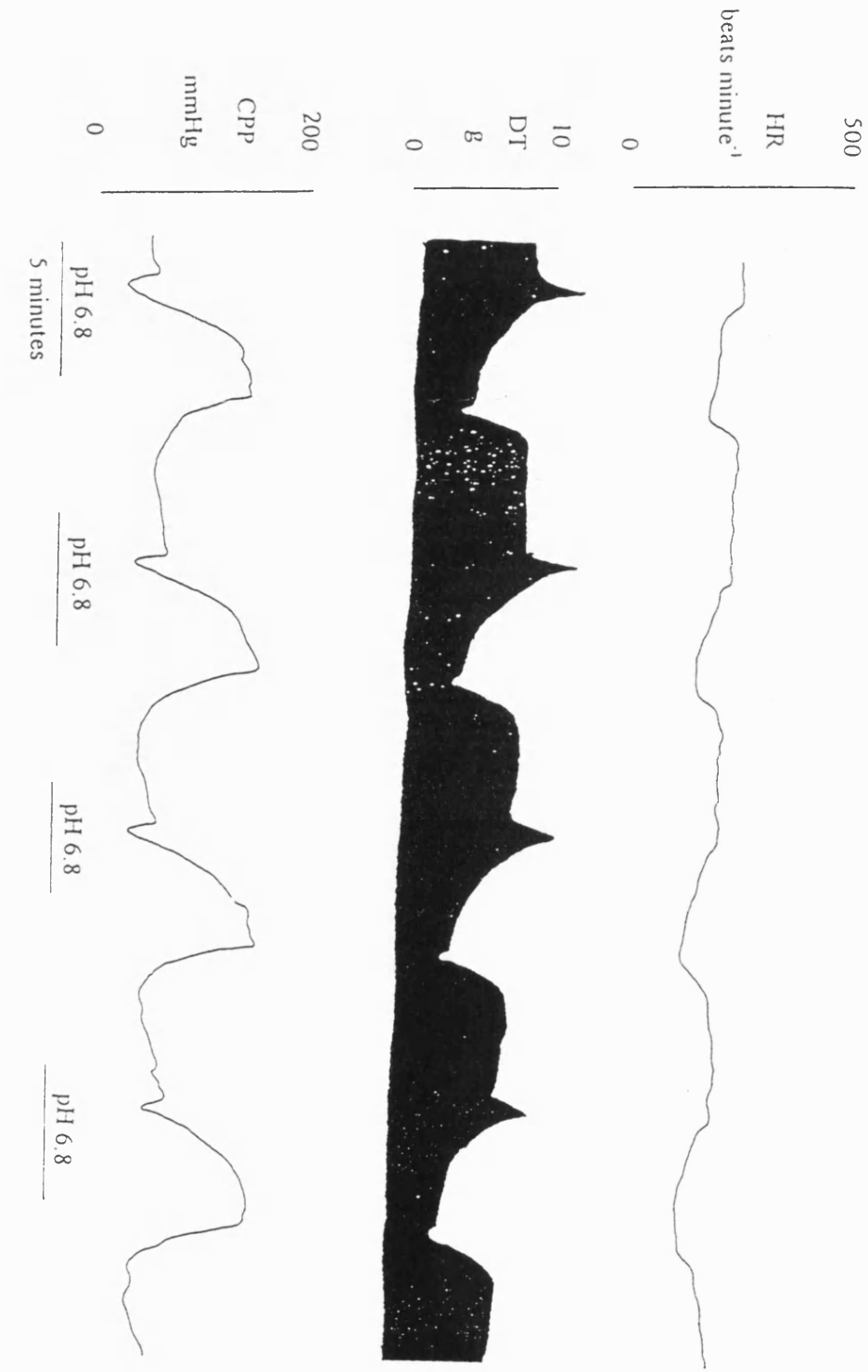


Figure R3: Representative experimental trace demonstrating the effects of a series of 4 applications of metabolic acidosis (pH 6.8) in the isolated rat heart. On the application of metabolic acidosis, there was a significant negative chronotropic and inotropic effect. Following an initial dilation, CPP underwent a sustained constriction. All effects were reversible and reproducible.

Metabolic acidosis (pH 6.8)

	Basal	Acidosis
HR	313±14	250±14*
DT	5.1±0.5	2.5±0.9*
CPP	89±10	164±10*

Respiratory acidosis (pH 6.8)

	Basal	Acidosis
HR	270±11	160±8*
DT	7.0±0.9	3.1±0.4*
CPP	82±3	176±10*

HEPES-buffered Tyrode (pH 6.8)

	Basal	Acidosis
HR	180±11	unrecordable
DT	2.8±1.0	0.5±0.1*
CPP	95±10	183±11*

Table R1: Table showing effects of metabolic and respiratory acidosis, plus HEPES Buffered Tyrode solution (pH 6.8) on coronary perfusion pressure (CPP mmHg), developed tension (DT g) and heart rate (HR beats minute^{-1}) in the isolated rat heart. Data represent mean ±s.e.m., n=4.

* $p<0.05$ compared to control

3.3 *Effect of Acidosis Applied At Different Flow Rates And Constant Head Of Pressure in the Rat Heart*

It has been demonstrated that shear stress can alter various endothelial cell factors including potassium currents (Olesen *et al.,* 1988), Ca^{2+} transients and intracellular Ca^{2+} levels (Schwarz *et al.,* 1992; Shen *et al.,* 1992). Therefore, investigation of acidosis-induced constrictions were performed at different flow rates. CPP was altered by modification of flow rate to 5 and 20ml.minute^{-1} and any effects compared to control constrictions seen at a flow rate of 10ml.minute^{-1}.

Reducing flow rates from 10ml.minute^{-1} to 5ml.minute^{-1}, and increasing the flow rate to 20ml.minute^{-1}, had no significant affect on contractility or heart rate. As observed using a flow rate of 10ml.minute^{-1}, metabolic acidosis (pH 6.8) still elicited a transient positive inotropic effect when applied at flow rates of 5 and 20ml.minute^{-1}. From a control value of 7.2±0.9g, DT increased by 2.3±0.6g at 10ml.minute^{-1}, 2.4±0.7 and 2.3±0.6g at flow rates of 5 and 20ml.minute^{-1}. After 5 minutes perfusion with metabolic acidosis (pH 6.8), DT fell from 7.2±0.9g to 3.1±0.4, 3.3±0.5 and 3.0±0.3 g at 10, 5 and 20 ml.minute^{-1}.

As can be seen from Table R2, at flow rates of 5 and 20ml.minute^{-1}, basal CPP was reduced and increased respectively compared with a flow rate of 10ml.minute^{-1}. Metabolic acidosis (pH 6.8), however, still elicited significant coronary constriction.

There was no significant difference between the negative chronotropic and inotropic effects of acidosis when applied using constant flow of 10ml.minute^{-1} and a constant head of pressure (100cm water above cannula). In control responses to acidosis at constant flow rate, DT decreased from 5.0±0.4 to 1.3±0.3 g ($p<0.05$) while HR fell from 283±16 to 220±19 beats minute^{-1}. Similar results were obtained using the constant head of pressure (see Figure R4). On switching to the constant head of pressure, there was no affect on DT while HR transiently increased by 38±9 beats minute^{-1}.

When metabolic acidosis was applied at a constant head of pressure, coronary flow significantly decreased, halving at the end of the 5 minute application period.

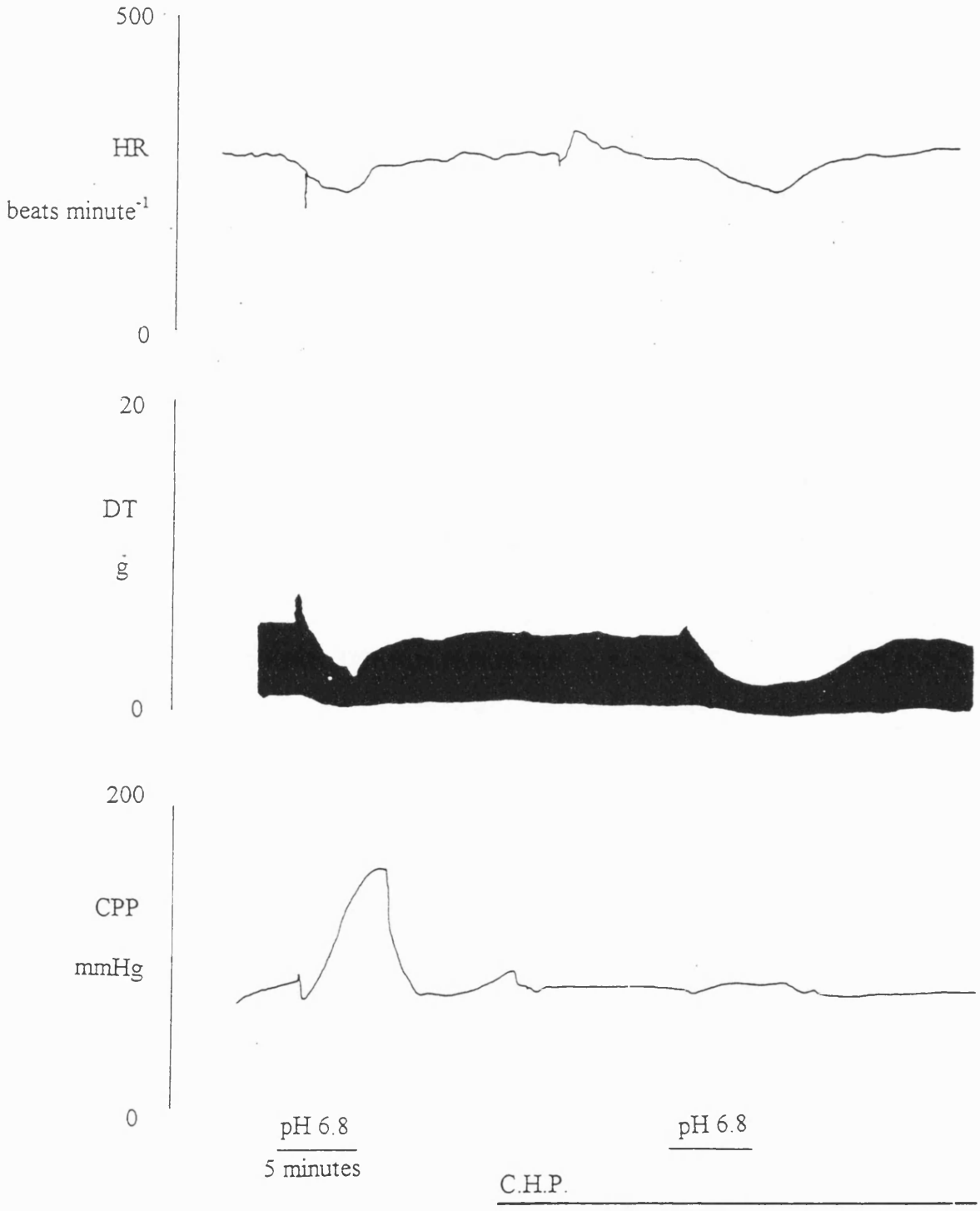


Figure R4: Typical trace showing effects of 5 minute application of metabolic acidosis (pH 6.8) at a constant flow rate (10ml.minute^{-1}) and under a constant head of pressure (CHP; 100cm above aortic cannula) in the isolated rat heart.

3.4 Coronary resistance

As mentioned above, since shear stress can modulate affect vascular tone, it was thought that in order to compare constant flow with constant head of pressure, measurement of coronary resistance would be a useful parameter to study.

Coronary resistance was calculated for the isolated rat heart under the various experimental conditions mentioned previously. Resistances were calculated under basal conditions at the three different flow rates (5, 10 and 20ml.minute^{-1}), and after 5 minutes of metabolic acidosis (pH 6.8) at the three flow rates. Using a constant flow system of 10ml.minute^{-1}, coronary resistance was 10.46±0.45 mmHg.gram.min.ml^{-1}. These figures increased steadily to 17.95±1.20 mHg.gram.min.ml^{-1} at the end of the 5 minute acidotic period. Similar increases were observed using flow rates of 5 and 20ml.minute^{-1} (see Table R2). It can be seen from Table R2 that as coronary flow rate is decreased (vasoconstriction under constant flow rate conditions) as a result of acidosis, so coronary resistance increased.

On switching to a constant head of pressure from a constant flow rate of 10 ml.minute^{-1} (reservoir 100cm above aortic cannula) flow rate fell steadily. After 5 minutes flow rate was 8.85±0.94ml.minute^{-1}. Following a 5 minute application of metabolic acidosis (pH 6.8) coronary flow rate decreased from the control value of 8.85±0.9 to 4.4±1.5ml.minute^{-1}.

Examination of the perfusion pressure record, when acidosis was applied using a constant head of pressure, showed only a modest effect on coronary perfusion pressure. Basal coronary perfusion pressure of 70±5mmHg increased to 74±5mmHg after 5 minutes perfusion with metabolic acidosis (pH 6.8). There were slight differences on reperfusion compared to constant flow rate. Despite perfusate pH returning to pH 7.4, flow rate remained reduced for 4 minutes.

In view of the results from these initial studies (negative chronotropic and inotropic effect and sustained increase in CPP), a more detailed study was performed to further characterise the acidosis-induced vasoconstriction since the myocardial have previously been reported.

CORONARY PERFUSION PRESSURE (mmHg)

Constant flow			Constant head of pressure	
Flow rate ($ml.minute^{-1}$)	**BASAL**	**ACIDOSIS**	**BASAL**	**ACIDOSIS**
5	38±1	95±5*		
10	80±7	158±11*	70±5	74±5
20	173±3	204±6*		

CORONARY RESISTANCE (mmHg.gram.min.ml^{-1})

Constant flow			Constant head of pressure	
Flow rate ($ml.minute^{-1}$)	**BASAL**	**ACIDOSIS**	**BASAL**	**ACIDOSIS**
5	10.0±0.2	18.3±3.9*		
10	10.8±1.2	17.96±1.2*	9.3±1.7	17.0±0.5*
20	10.8±0.7	13.1±01.5*		

Table R2: Table showing basal and metabolic acidotic (pH 6.8) coronary perfusion pressure (CPPmmHg) and coronary resistance measurements (mmHg.gram.min.ml^{-1}) obtained at constant flow rates (5, 10 and 20$ml.minute^{-1}$) and constant head of pressure in the isolated rat heart.

Data are mean±s.e.m., n=4.

* $p<0.05$ compared to basal level

3.5 Species And Tissue Variation In Vascular Response To Acidosis

In order to investigate whether the acidosis-induced coronary constriction observed in the isolated rat heart would occur in other vascular preparations, other species and tissues were used to compare the effects of metabolic acidosis (pH 6.8).

3.5.1 Isolated Guinea Pig Heart

Following equilibration in Krebs-Henseleit solution at a flow rate of 10ml.minute^{-1} (pH 7.4), CPP was 63±5mmHg, DT 5.0±0.5g and HR 210±6beats minute^{-1}. On changing pH to 6.8, there was an initial transient positive effect on DT of 0.6±0.1g. As illustrated in Figure R5, after a 5 minute application of acidic Krebs-Henseleit solution (pH 6.8), CPP decreased to 44±3mmHg ($p<0.02$), DT to 2.0±0.3g ($p<0.05$) and HR to 180±9 ($p<0.05$) beats minute^{-1} ($p<0.05$). All effects were reversible on return to pH 7.4.

As in the experiments using Krebs-Henseleit buffered solutions, similar effects were seen using hypercarbic-induced acidosis and HEPES-Buffered Tyrodes. In experiments using respiratory acidosis (pH 6.8), control (pH 7.4) CPP was 58±7mmHg, DT 5.3±0.8g and HR 284±9beats minute^{-1}. In contrast to metabolic acidosis (pH 6.8), there was no initial transient positive inotropic effect on perfusion with the acidotic solution. After 5 minutes perfusion with respiratory acidosis (pH 6.8), CPP decreased to 47±6mmHg, DT to 2.2±0.5g and HR to 229±6beats minute^{-1}.

On changing from Krebs-Henseleit solution to HBT solution (pH 7.4), there were sustained decreases in all parameters measured. CPP decreased from 64±8 to 53±4mmHg, DT from 5.1±0.5 to 3.4±0.3g and HR from 204±7 to 136±15beats minute^{-1} (all $p<0.05$). Further decrease in these parameters reversed on perfusion with acidotic HBT. CPP decreased to 43±3mmHg, DT to 2.1±0.2g and HR to 120±8beats minute^{-1}.

Figure R5: Representative experimental trace demonstrating the effects of 5 minute applications of metabolic acidosis (pH 6.8) on the isolated guinea-pig heart. Similar to the observations in the rat heart, there was a negative chronotropic and inotropic effect in the guinea pig heart. In contrast, however, coronary perfusion pressure underwent a sustained dilation.

3.5.2 *Isolated Porcine Coronary Artery*

There was no effect of metabolic acidosis (pH 6.8) on the resting tension of 1g in isometrically mounted porcine coronary ring preparations. In order to investigate the effects of acidosis in pre-constricted preparations, tissues were exposed to phenylephrine or K^+. Surprisingly, no constriction was observed in response to phenylephrine (0.1-10μM). In contrast to this, preparations were responsive to K^+. A dose response curve to K^+ was constructed and a total K^+ concentration of 42mM was chosen as a standard concentration which gave approximately 70% maximum response. Figure R6 is a representative experimental trace showing the effects of metabolic acidosis (pH 6.8) on a K^+ depolarized preparation. From the control value of 2.3±0.3g at pH of 7.4, metabolic acidosis (pH 6.8) caused tension to decrease to 1.7±0.2g ($n=5$; $p<0.05$). This relaxant affect reversed on restoration of pH 7.4.

3.5.3 *Isolated Rat Aorta*

As in the original study (Furukawa *et al.* 1996), aortic rings reversibly constricted in response to 60mM KCl (osmotically balanced). 5 minutes after adding K^+, tension increased from 1 to 1.63±0.09g ($p<0.05$). This effect was reproducible for three subsequent challenges. In contrast to K^+-constricted pig coronary arteries, the rat aorta did not relax when metabolic acidosis (pH 6.8) was applied. A similar lack of effect was observed when extracellular pH was altered by addition of HCl, the method used by Furukawa *et al.* (1996).

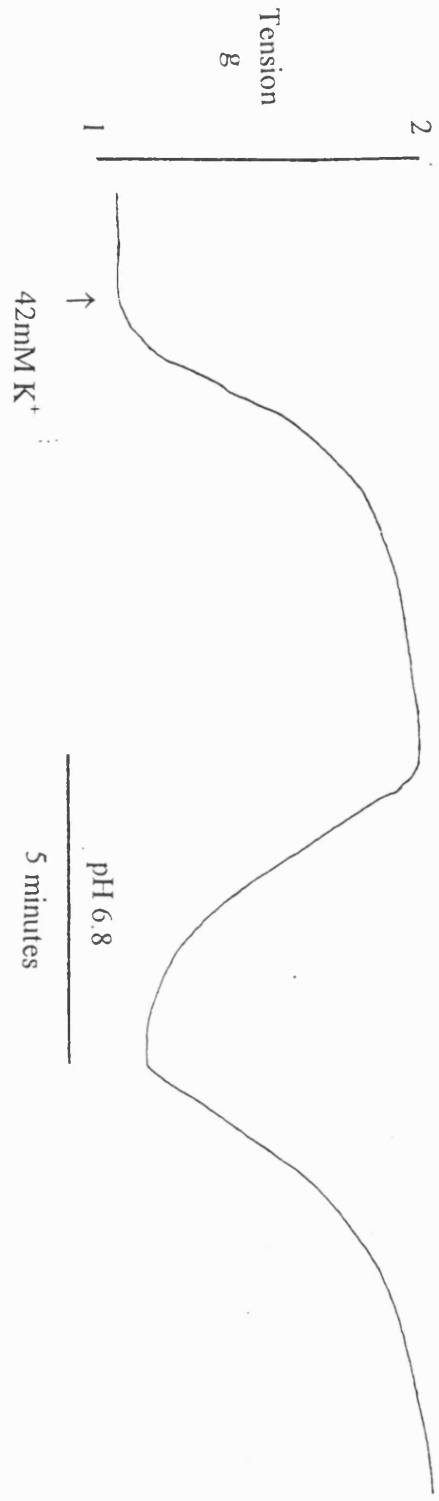


Figure R6: Experimental trace showing effects of metabolic acidosis (pH 6.8) on a isometrically mounted pre-constricted (42mM K^+) porcine coronary artery ring. Exposure to 42mM K^+-containing solution (pH 7.4) constricted the porcine coronary artery ring. Switching the solution pH (in the continued presence of 42mM K^+) reversibly dilated the preparations.

3.5.4 *Isolated Perfused Superior Mesenteric Bed*

Similar experiments, as performed on the isolated heart, were undertaken on basal and pre-constricted (phenylephrine 10μM and 40mM K^+) superior mesenteric beds to contrast and compare the effects of alterations in pH on a systemic vascular resistance bed with those of the coronary circulation.

Neither metabolic or respiratory acidosis (both pH 6.8) had any affect on basal mesenteric perfusion pressure. When pre-constricted, however, the results were dependent on the agent used. In general, K^+ pre-constricted preparations were unresponsive to experimental pH changes whereas phenylephrine pre-constricted mesenteric beds were responsive.

Figure R7 shows data from experiments performed using pre-constricted superior mesenteric beds (both phenylephrine and K^+) and the effects of 5 minute perfusion of metabolic and respiratory acidosis (both pH 6.8). It can be seen that K^+ pre-constricted mesenteric beds were unaffected by acidosis of both types. In phenylephrine constricted preparations the effects of acidosis were dependent on the procedure used to alter pH. Perfusion with respiratory acidosis (pH6.8) increased mesenteric perfusion pressure in phenylephrine constricted preparations. This constriction reversed towards basal levels during the 5 minute perfusion with the acidotic solution. Control levels were reached within 10 minutes. In contrast, metabolic acidosis (pH6.8) produced a sustained dilation of phenylephrine constricted preparations. On washout, there was a re-bound constriction which returned to control levels within 10 minutes

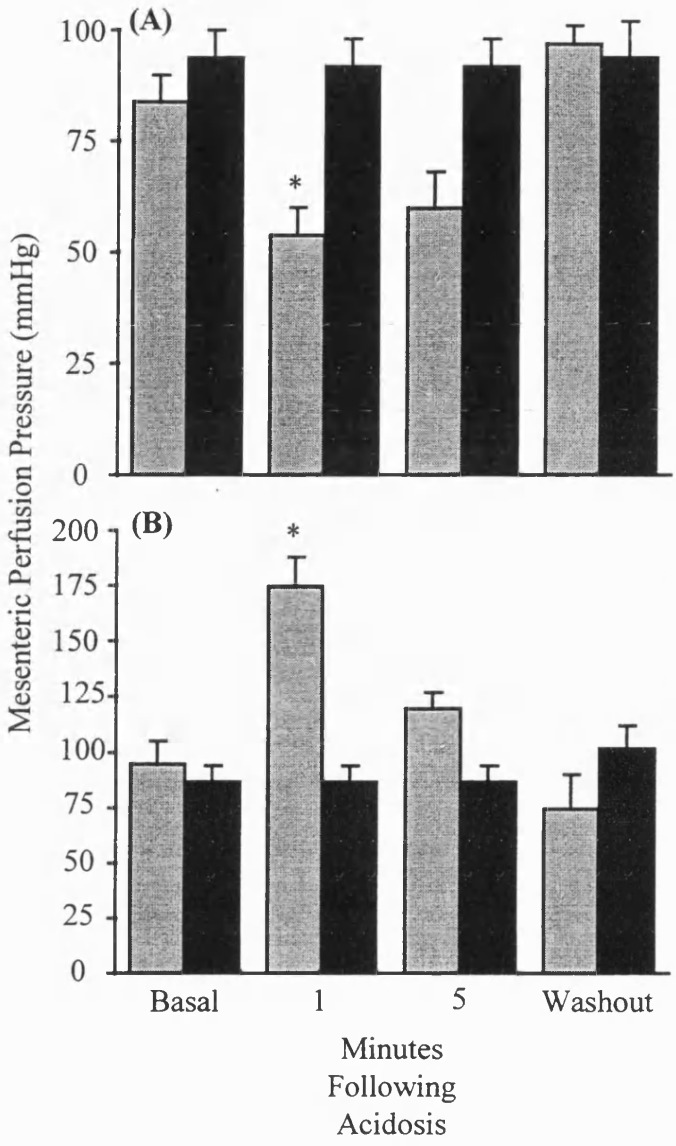


Figure R7: Cumulative histograms showing the effects of metabolic (A) and respiratory (B) acidosis (both pH 6.8) on phenylephrine (10μM; grey bars)-and potassium (40mM; dark bars)- pre-constricted rat superior mesenteric beds. Data mean ± s.e.m., n=6. *$p<0.05$ compared to basal levels.

It can be seen that K^+-constricted preparations were generally unresponsive whilst phenylephrine-constricted preparations dilated in response to metabolic acidosis and transiently constricted in response to respiratory acidosis (both pH 6.8).

3.6 Alterations Of Intracellular pH Only

3.6.1 Isolated Rat Heart

It is possible, by the use of weak acids and salts, to selectively change intracellular pH whilst maintaining extracellular pH constant (Taggart *et al.* 1994). For this purpose, butyric acid (sodium salt) and NH_4Cl were used to examine the effects of intracellular pH changes alone on vascular tone and a comparison made with situations where extra- & intracellular pH was reduced. The effects were examined in rat hearts and pre-constricted mesenteric beds.

3.6.1.1 Ammonium chloride

Measurements of intracellular pH using this compound have revealed that on perfusion intracellular alkalosis occurs which is maintained in the presence of the compound. On washout, intracellular pH falls below resting levels before being restored to normal (Taggart *et al.* 1994).

Contained within figure R8 are data from experiments where 10mM NH_4Cl was perfused through isolated rat hearts to study its effects.

It can be seen that HR is not significantly affected by the addition or removal of NH_4Cl. In contrast, DT was decreased only on the washout of NH_4Cl. CPP was significantly decreased by the addition of NH_4Cl and showed a re-bound increase during NH_4Cl washout.

The alterations on addition of NH_4Cl (*i.e.* increase in DT and decrease in CPP) are similar to the transient effects of metabolic acidosis, while the effects observed on removal (decrease in DT and increase in CPP) are similar to the sustained effect of metabolic acidosis.

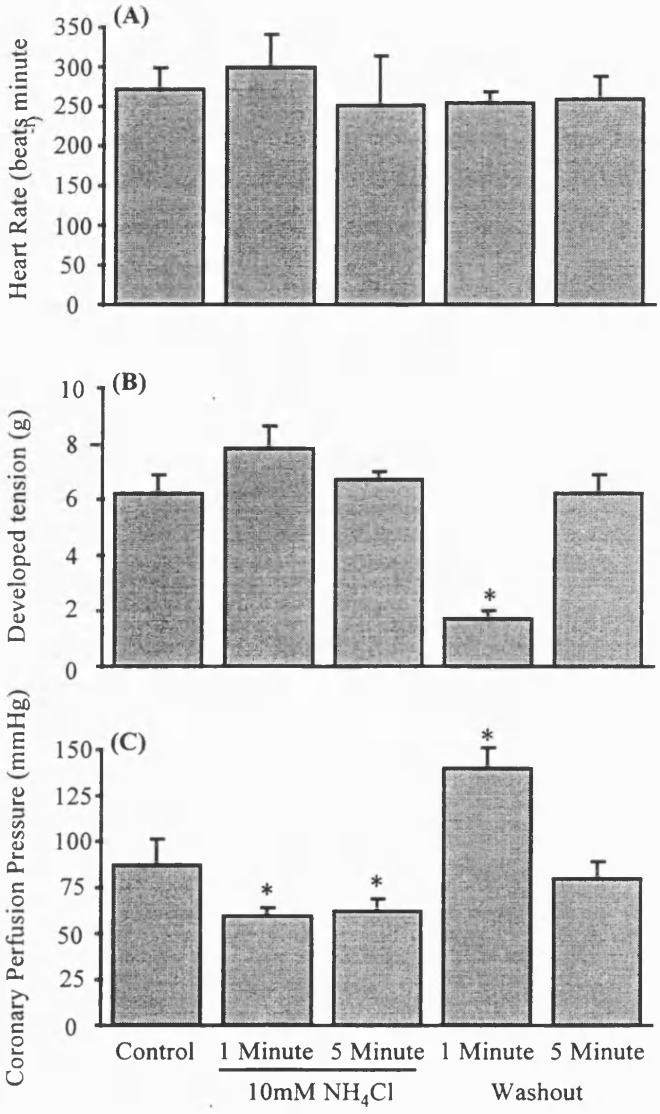


Figure R8: Cumulative histograms showing the effects of 5 minute perfusion of NH_4Cl followed by a 5 minute wash-out period on heart rate (A), developed tension (B) and coronary perfusion pressure (C) in the isolated rat heart. Data mean ± s.e.m., n=5.

$p<0.05$ compared to basal

There was no alteration in HR while DT significantly decreased on the removal of NH_4Cl. CPP decreased in the presence of NH_4Cl but significantly increased on removal.

3.6.1.2 *Butyric acid*

In response to a 5 minute application of butyric acid (1, 10 and 50mM), there was no significant change in HR, while DT and CPP were both decreased in a concentration-dependent manner. HR, 290±9beats minute^{-1} was reduced to 275±14beats minute^{-1} at a concentration of 50mM. DT transiently decreased in response to butyric acid. In response to butyric acid (1, 10 and 50mM), DT control value of 9.4±1.2g decreased to 9.1±0.6, 5.9±0.8 ($p<0.05$) and 3.8±0.3g ($p<0.05$) initially on perfusion with recovery to 9.4±1.1, 7.4±0.4 and 5.3±0.9g ($p<0.05$) at the end of the 5 minute perfusion. CPP decreased in response to all concentrations of butyric acid used from control value of 88±8mmHg, after 5 minute it was 84±9, 59±3 ($p<0.05$) and 53±8 ($p<0.05$) mmHg.

3.6.2 *Isolated Superior Mesenteric Bed*

3.6.2.1 *Ammonium chloride*

A similar pattern to that observed with metabolic acidosis was seen when NH_4Cl was applied and removed to produce intracellular alkalosis and acidosis. Preparations constricted with K^+ were unresponsive when compared to phenylephrine to NH_4Cl. In contrast, the addition of NH_4Cl to phenylephrine constricted preparations caused a significant decrease in MPP which partially recovered at the end of the 5 minute perfusion period. On washout, MPP significantly increased, returning to control levels after 10 minutes.

3.6.2.2 Butyric acid

K^+ constricted preparations were unresponsive to intracellular acidosis induced by the application of 10mM butyric acid. In contrast, MPP in phenylephrine constricted preparations significantly increased on the application of 10mM butyric acid, returning towards control after 5 minutes, although remaining above control levels (figure 10B).

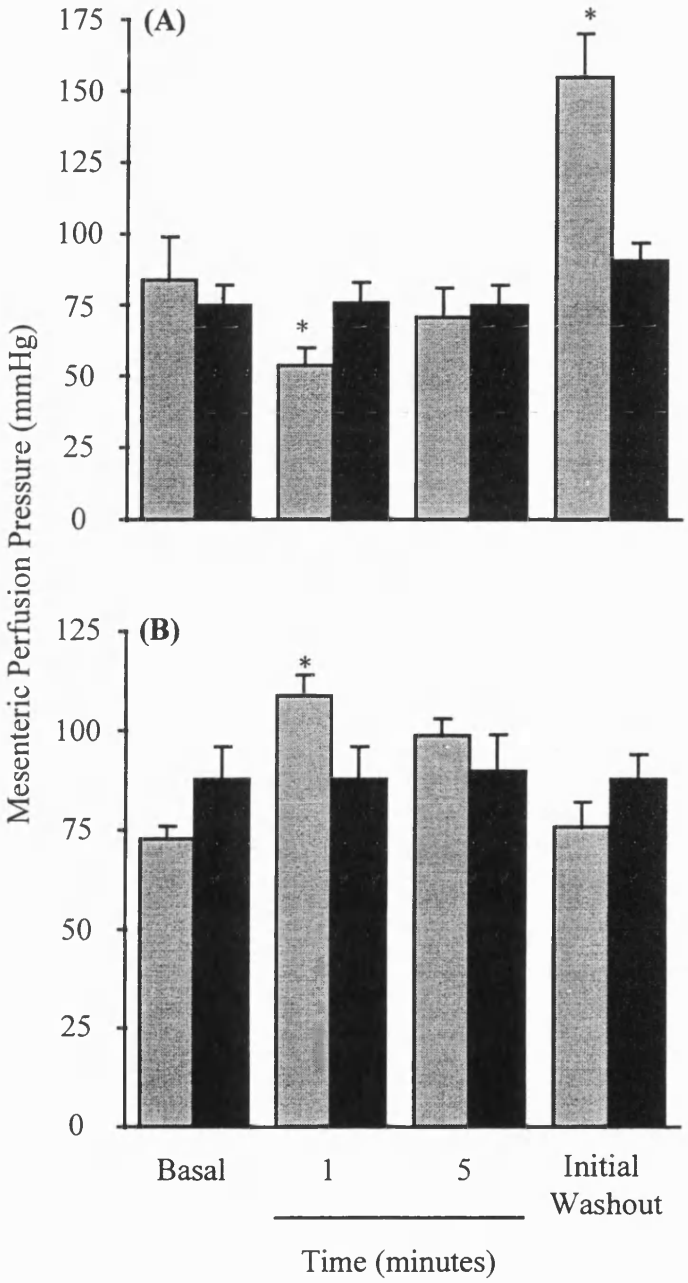


Figure R9: Cumulative data showing the effects of 5 minutes perfusion of NH_4Cl (A) and butyric acid (B) in phenylephrine (10μM; light bars) and potassium (40mM; dark bars) constricted rat superior mesenteric beds. Data mean ± s.e.m., n=5

* $p<0.05$ compared to control

K^+-constricted preparations were unresponsive to both manoeuvres while phenylephrine-constricted preparations constricted on removal of NH_4Cl and the application of butyric acid.

3.7 Does Cardiac Contraction Modulate Acidosis-Induced Constriction ?

As cardiac work is a major factor regulating coronary flow, the decrease in DT, HR and work load produced by acidosis could indirectly lead to an increase in CPP. In order to investigate this, experimental manoeuvres were performed to reduce or abolish cardiac contraction prior to the application of acidosis. Two methods were employed to reduce cardiac contraction. Namely:

3.7.1 Pacing

The isolated rat heart is known to undergo a phenomenon known as a negative staircase effect (Borzak *et al.* 1991). In this, increases in the rate of electrical stimulation cause a reduction in the force of cardiac contraction. When isolated rat hearts were stimulated (6-16Hz.), there was a fall in DT while there was an increase in resting tension from basal levels of 2g. At a stimulation rate of 16Hz. (960 beats minute^{-1}), DT fell significantly to 0.67±0.1g ($p<0.05$) while resting tension increased from 2.0 to 4.3±0.4g ($p<0.05$).

Interestingly, coronary perfusion pressure was only transiently affected by pacing. Between the pacing range used in the experiments (6-16Hz.), a transient dilation of 10-15mmHg was observed on each increment of the stimulation frequency. CPP returned to control levels within 5 minutes of the increase of stimulation. It can be seen from figure R10A that under these conditions, acidosis produced a constriction similar in magnitude to that seen in unpaced control hearts.

3.7.2 Hyperkalemia

In this series of experiments, total [K_o^+] was increased from 5.9 to 15mM in order to suppress cardiac contraction. Control coronary constriction in response to metabolic acidosis (pH 6.8) was from 66±5 to 129±15mmHg ($p<0.05$). On perfusion with hyperkalemic Krebs-Henseleit solution, cardiac contraction immediately ceased. There was a transient dilation with CPP falling to 56±6mmHg, recovering to control level within 10 minutes. It can be seen from figure R10B that there was no significant difference between acidosis-induced coronary constrictions in control, hyperkalemic and recovery challenges.

The results obtained with the pacing experiments and hyperkalemia would suggest that the coronary vasoconstriction induced by metabolic acidosis (pH 6.8) is independent of the myocardial depressant effects of acidosis.

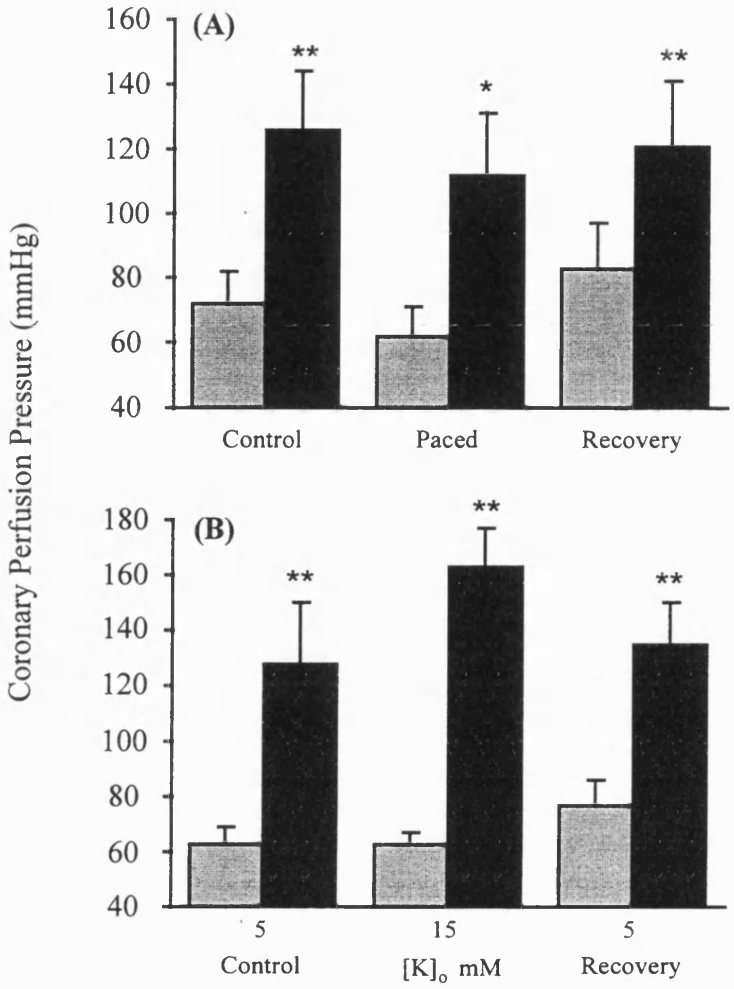


Figure R10: Cumulative histograms showing the effects of decreasing cardiac contraction induced by pacing (A) and hyperkalemia (15mM K^+; B) on basal CPP (grey histograms) and CPP response to metabolic acidosis (pH 6.8; dark histograms) n the rat heart. Data are mean ± s.e.m., n=5. *$p<0.05$ compared to control **$p<0.02$ compared to control

It can be seen that the metabolic acidosis-induced coronary constriction was unaffected by protocols which decreased/abolished cardiac contraction (pacing and hyperkalemia).

3.8 *Inhibitors Of Nitric Oxide Synthase And Cyclo-oxygenase Pathways: The Effect on Acidosis-Induced Increase In CPP.*

The previous section demonstrated that acidosis causes coronary vasoconstriciton independent of myocardial effects. A further possibility for the observed acidosis-induced constrictions in the isolated rat heart include either, enhanced production of an endogenous vasoconstrictor, or, attenuation of an endogenous vasodilator agent. It is known that adenosine, nitric oxide, prostaglandins and thromboxanes are implicated in the regulation of coronary flow. In order to investigate the possibility that changes in the levels of one of these mediators was involved, the following drugs were used. The adenosine antagonist 8-PT was used to investigate the possible role of adenosine, the cyclo-oxygenase enzyme inhibitor indomethacin, to investigate cyclo-oxygenase products; the nitric oxide synthase inhibitor, N_{ω}-nitro-L-ARGININE (NO-ARG) to investigate the role of nitric oxide and the dual thromboxane A_2 synthase inhibitor and receptor antagonist ZD 1542 to examine the possible role of thromboxane A_2.

To examine the role of adenosine, a control acidosis response was initially determined. CPP was initially 84±9mmHg and on application of metabolic acidosis (pH 6.8) transiently fell by 26±8mmHg on the application of acidosis before increasing to 159±11mmHg ($p<0.05$) after 5 minutes of acidosis. Control DT transiently increased from 8.5±0.9g by 1.9±0.5g on the application before acidosis before decreasing to 3.4±0.6g at the end of the 5 minute acidotic period. After returning to pH 7.4 a control response to a 100 nmoles bolus dose of adenosine was determined. This significantly reduced CPP by 28±5mmHg. Following 15 minutes perfusion with 10μM of the adenosine antagonist 8-PT, there was no effect on DT or HR, however, CPP

significantly increased from 84±9 to 125±6mmHg ($p<0.05$). In the presence of 8-PT, administration of adenosine (100nmole) had no effect on CPP. In contrast, on the application of metabolic acidosis CPP transiently dilated by 24±6mmHg and underwent a sustained increase to 154±9mmHg ($p<0.05$) *i.e.* 8-PT did not affect the acidosis-induced increase in CPP.

The cyclo-oxygenase enzyme utilises arachidonate from cell membrane phospholipids to manufacture various vasoactive substances. One of these is thromboxane A_2, a potent vasoconstrictor. Acidosis could possibly be stimulating the production of such a vasoconstrictive agent generated by the cyclo-oxygenase pathway. However, as can be seen from Table R3, perfusion for 15 minutes with indomethacin (10μM) had no significant effect on any of the basal experimental parameters, or the acidosis-induced changes in HR, DT or CPP.

Further evidence for thromboxane A_2 not being involved in the response to acidosis was gained using ZD1542. This compound is a dual thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor (Brownlie *et al.* 1993). In the concentration range 0.01 to 10μM, ZD 1542 had no effect on basal parameters or responses due to metabolic acidosis (pH 6.8). In control responses to metabolic acidosis, HR fell from 318±8 to 245±15 beats minute^{-1}, DT fell from 9.1±1.4 to 4.5±1.2 g and CPP increased from 78±16 to 165±10 (all $p<0.05$). In the presence of 10μM ZD 1542, HR fell from 310±9 to 240±12, DT from 8.5±1.1 to 4.1±0.5, while CPP increased from 82±9 to 175±13 (all $p<0.05$). There was also no effect of the solvent control using 1:1000 DMSO.

Endothelium derived relaxing factor, or nitric oxide (NO), is known to have a relaxant effect on the coronary circulation of the isolated rat heart (Randall 1995). NO-ARG is an active competitor of L-arginine, the natural substrate for the nitric oxide synthase enzyme. Results obtained after 15 minutes perfusion with NO-ARG (100μM) showed that basal CPP significantly increased in response to the compound from 81±3 to 119±10mmHg ($p<0.05$). However, inhibition of NO synthesis had no effect on the transient dilation observed or sustained constriction induced by acidosis (see table R3).

	CONTROL	INDO (10μM)	CONTROL	NO- ARG (100μM)
BASAL HR	303±16	308±6	282±11	287±14
ACIDOSIS HR	258±11*	243±14*	230±13*	220±13*
BASAL DT	10.3±0.3	9.9±1.0	7.8±0.9	7.3±1.0
ACIDOSIS DT	5.3±1.0*	4.8±0.6*	3.5±0.9*	3.2±0.4*
BASAL CPP	94±2	96±10	81±3	119±10#
ACIDOSIS CPP	171±11*	180±14*	166±7*	176±6*

Table R3: Data representing the effects of 15 minutes perfusion of indomethacin (10μM) and NO-ARG (100μM) on basal and acidosis-induced changes in heart rate (HR; beats minute^{-1}), developed tension (DT; g) and coronary perfusion pressure (CPP; mmHg) in the isolated rat heart. Data are mean ± s.e.m., n=6. * $p<0.05$ compared to basal control # $p<0.05$ compared to basal CPP.

Data presented here demonstrates that basal CPP constricted in the presence of NO-ARG, there was no effect of NO-ARG or indomethacin on the metabolic acidosis-induced changes in HR, DT or CPP.

3.9 *Effects Of An α-adrenoceptor Antagonist On Acidosis-Induced Increase In CPP*

Decreases in pH have been shown to cause release of vasoactive substances from nerve endings (Franco-Cerada *et al.* 1994). To investigate the possibility that acidosis could be releasing stored catecholamines to act on α-adrenoceptors and cause coronary constriction, phentolamine was used. In control responses to 10nmoles boluses of phenylephrine CPP increased from 94±8 to 129±6mmHg($p<0.05$). After 10 minutes perfusion with 10μM phentolamine, the increase in CPP in response to the same bolus dose of phenylephrine was attenuated, increasing to 98±3mmHg ($p>0.05$). These results are represented in figure R11(B). Therefore, this concentration of phentolamine and a contact time of 10 minutes is having a blocking effect on α-adrenoceptors on vascular smooth muscle.

Phentolamine had no effect on DT changes induced by metabolic acidosis. Basal DT, 8.3±0.8g, transiently increased to 10.1±0.6g before decreasing to 3.7±0.5g ($p<0.05$). After 10 minutes application of phentolamine (10μM), basal DT was 8.1±0.7g, on the application of metabolic acidosis, DT transiently increased to10.3±0.8g before undergoing a sustained decrease to 3.3±0.8g ($p<0.05$).

Phentolamine also had no effect on any of the vascular effects of metabolic acidosis. In response to acidosis control CPP decreased by 27±9mmHg before increasing to 168±16mmHg ($p<0.05$). In the presence of phentolamine (10μM), CPP decreased by 25±6mmHg and increased to 159±9mmHg ($p<0.05$). These data are represented in Figure R11(A)).

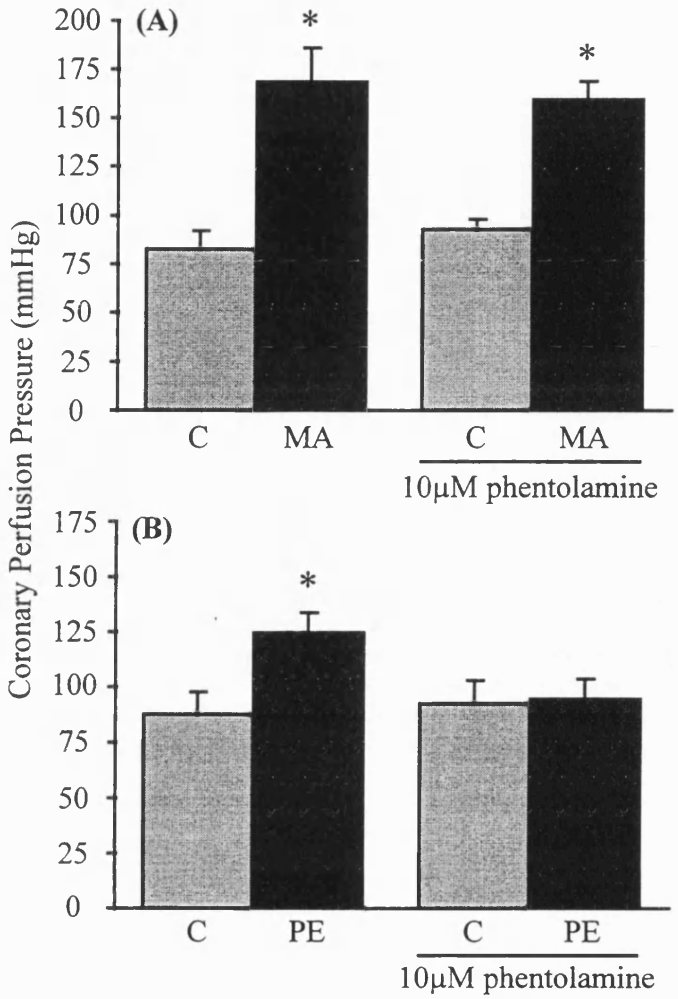


Figure R11: Histograms showing effects of phentolamine (10μM) on a) control CPP (C) and CPP in response to metabolic acidosis (MA), b) and 10nmole bolus dose of phenylephrine (PE) in the isolated rat heart. Data are mean ± s.e.m., n=5. * $p<0.05$

The concentration of phentolamine used completely inhibited the vascular effects a bolus dose of phenylephrine. It did not, however, have any effects on basal or metabolic acidosis-induced changes in CPP.

3.10 Role Of Extracellular Ca^{2+} In Acidosis-Induced Increase In CPP.

Acidosis produced by the three experimental procedures (decrease in $[HCO_3^-]$, increase in pCO_2 or using HEPES-buffered Tyrode) caused reversible coronary constriction. As commented on earlier, vascular smooth muscle contraction is highly dependent on the levels of cytosolic Ca^{2+}. One of the principal routes for entry of external Ca^{2+} into vascular smooth muscle cells is through specific channels located in the cell membrane. There is a growing family of Ca^{2+} channels, but the primary member and most important in terms of smooth muscle cell remains the voltage-sensitive L-type Ca^{2+} channel. To further elucidate its possible role in acidosis-induced coronary constriction, specific L-type Ca^{2+} channel antagonists and Ca^{2+}-free Krebs-Henseleit solution were employed in attempt to attenuate the CPP changes brought about acidosis.

3.10.1 Ca^{2+}-free Krebs-Henseleit solution

Control constriction in response to metabolic acidosis (pH 6.8) in normal Krebs-Henseleit solution increased CPP from 90±8 to 160±5mmHg ($p<0.05$). Immediately on switching to Ca^{2+}-free Krebs-Henseleit solution, cardiac contractility ceased. Surprisingly, CPP only fell to 87±11mmHg after 5 minutes. Under these conditions, when acidosis was re-applied the vasoconstriction previously observed was significantly attenuated, increasing from 87±11 to 94±11mmHg ($p<0.05$ compared to Ca^{2+} control ANOVA). Figure 12 describes the cumulative data from these experiments.

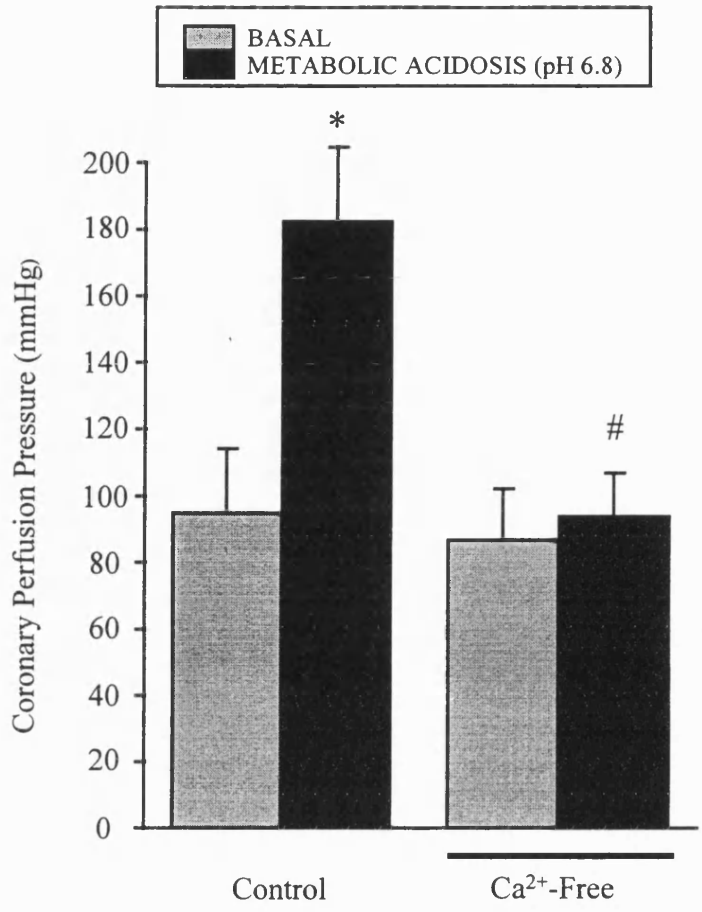


Figure R12: Histograms showing the effects of nominally Ca^{2+}-free Krebs-Henseleit solution on basal CPP and the response of the isolated rat heart to 5 minute application of metabolic acidosis (pH 6.8). Data are mean ± s.e.m., n=4. * $p<0.05$ compared to basal control; # $p<0.05$ compared to acidotic control constriction (oneway ANOVA).

It can be seen that, surprisingly, basal CPP is unaffected by the removal of extracellular Ca^{2+} whereas the metabolic acidosis-induced constriction was completely inhibited.

3.10.2 *Effect of Ca^{2+} Channel Antagonists On Acidosis-Induced Increase In CPP*

A series of Ca^{2+} channel antagonists were used to further investigate the role of Ca^{2+} entry via L-type Ca^{2+} channels on the acidosis-induced constrictions in the isolated rat heart.

In a series of experiments performed with Krebs-Henseleit solution nifedipine (1-100nM) had no effect on HR. In response to increasing concentrations of nifedipine, DT underwent a concentration-dependent decrease. DT decreased from 7.8±0.5g to 4.75±0.3g in response to 5 minute application of 100nM nifedipine. DT underwent a further sustained decrease in response to acidosis, falling to 2.3±0.3g after 5 minutes.

Nifedipine (1-100nM) caused a concentration-dependent decrease in CPP and inhibited the metabolic acidosis-induced increase in CPP, this was significant at a concentration of 100nM ($p<0.05$ compared to control ANOVA; see figures R13 and R14).

Data presented in Figure R15 are from experiments performed using the highest concentration of nifedipine (100nM) and hypercarbic acidosis and HEPES-buffered Tyrode (pH 6.8). These showed that the coronary constrictions in the isolated rat heart to both of these acidotic solutions are significantly attenuated by nifedipine (100nM).

Similar attenuations of the acidosis -induced increase in CPP were observed using two other Ca^{2+} channel antagonists. Amlodipine, another dihydropyridine, caused similar effects to nifedipine. Control constrictions in response to metabolic acidosis (pH 6.8) were significantly attenuated in the presence of amlodipine (100nM). The control

response to acidosis increased basal CPP from 85±9 to 171±14 mmHg ($p<0.05$). Amlodipine (100nM) significantly reduced basal CPP to 51±7 mmHg ($p<0.05$) and the acidosis-induced constriction to 59±8mmHg ($p<0.05$ ANOVA). Similar results were also obtained using verapamil (1μM). CPP increased from 78±8mmHg to 167±11mmHg ($p<0.05$) in a control constriction to metabolic acidosis. After 5 minute perfusion with verapamil(1μM) basal CPP fell to 48±6mmHg, only increasing to 57±8mmHg after 5 minute perfusion with metabolic acidosis in the presence of verapamil.

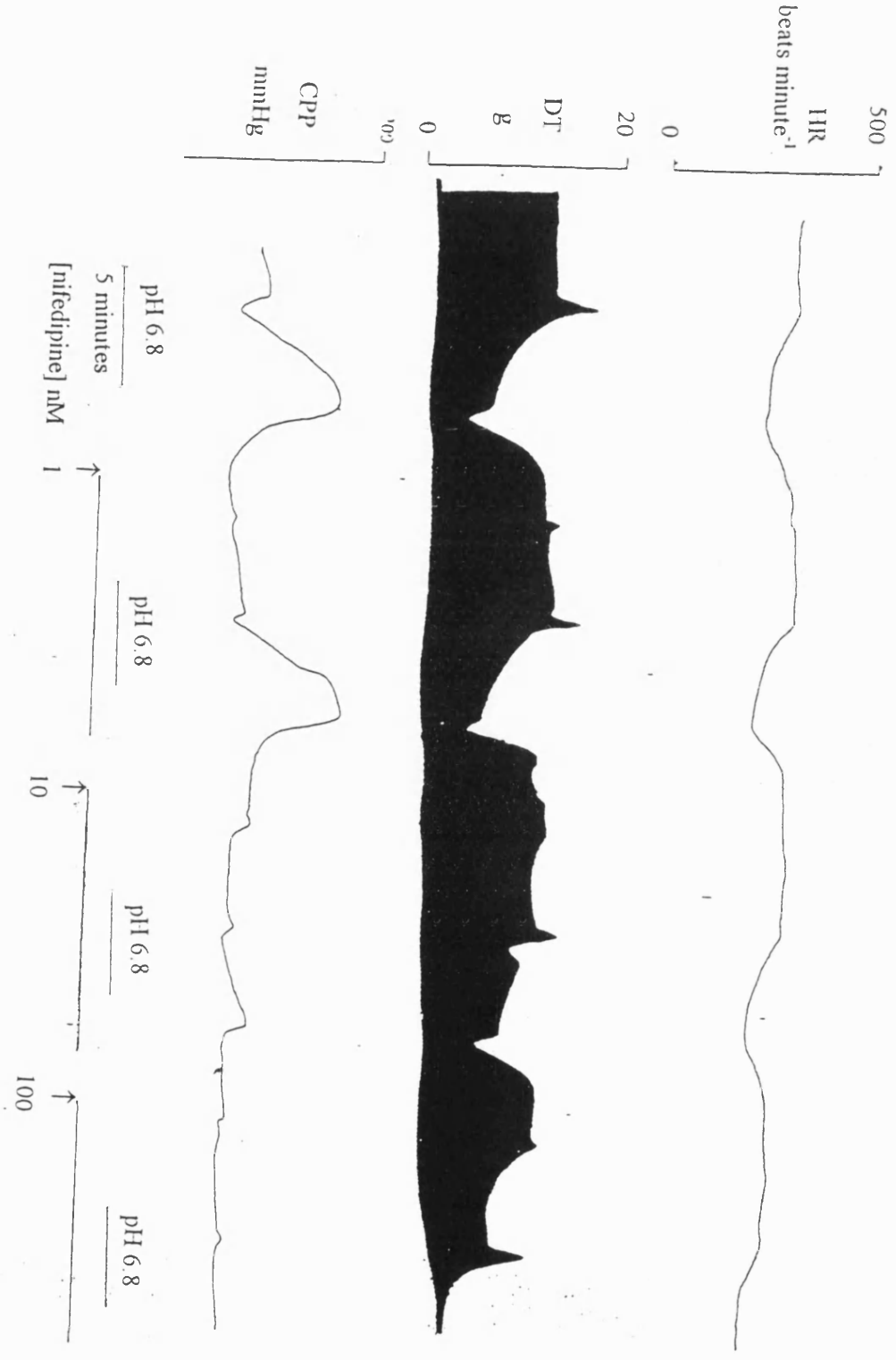


Figure R13: Representative experimental trace showing effects of nifedipine (1 - 100nM) on the isolated rat heart and its response to 5 minute application of metabolic acidosis (pH 6.8)

This figure and figure R14 demonstrate that increasing concentrations of the L-type Ca^{2+} channel blocker nifedipine decreased basal CPP but also inhibited the metabolic acidosis-induced coronary constriction.

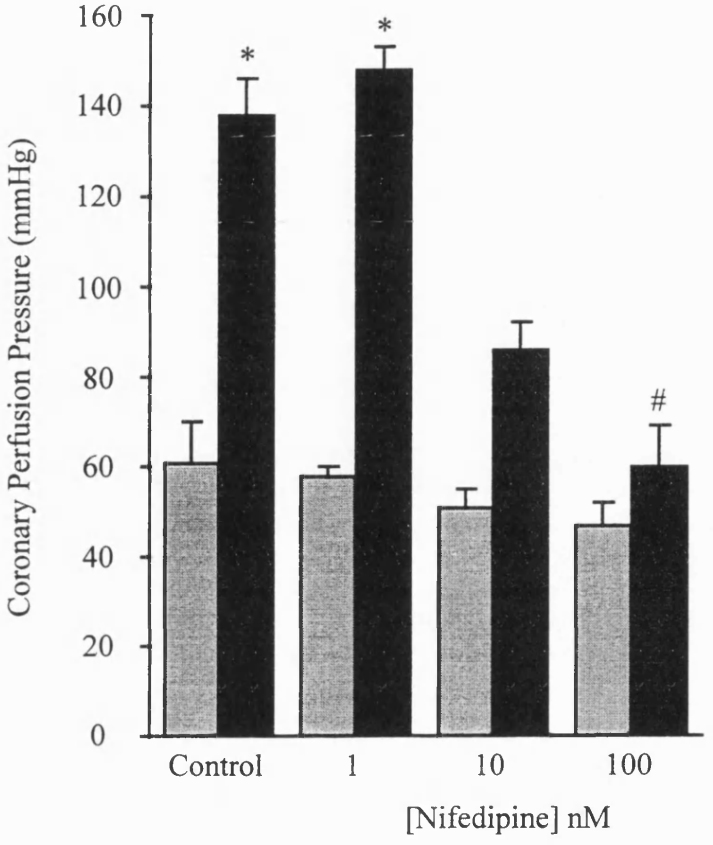


Figure R14: Cumulative data representing the effects of nifedipine (1-100nM) on the constriction in response to metabolic acidosis (pH 6.8) in the isolated rat heart.

Grey - basal CPP

Black - acidosis CPP

Data mean ± s.e.m., n=5.

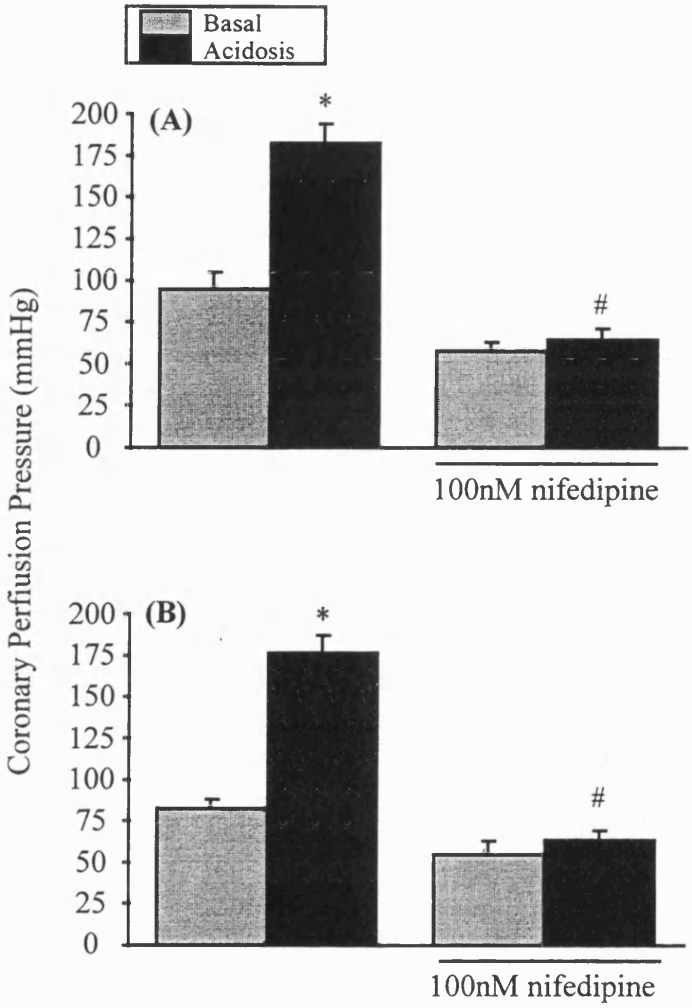


Figure R15: Cumulative histograms showing the effects of nifedipine (100nM) on basal CPP and the CPP response to acidosis (pH 6.8) in HEPES buffered Tyrode (A) and (B) hypercarbic acidosis and the attenuation by nifedipine in the isolated rat heart.

Data mean ± s.e.m., n=6. * $p<0.05$ compared to basal; # $p<0.05$ compared to basal

When acidosis (pH 6.8) was applied using respiratory acidosis and HEPES-Buffered Tyrode, nifedipine still inhibited the coronary constriction obtained with acidosis.

3.11 *Effects Of Vasodilation On Acidosis-Induced Increase in CPP*

Results presented earlier using the Ca^{2+} channel antagonists nifedipine, amlodipine and verapamil demonstrated that these compounds relaxed coronary vessels and attenuated the acidosis-induced coronary constriction. It is possible that the attenuation was simply due to a physiological antagonism. Therefore, to act as a control and to investigate whether the attenuation occurred as a result of the observed vasodilation, CPP was reduced using two further manoeuvres: hypoxia, and the vasodilator isopropyl palmitoyl carnitine (P1Pi).

3.11.1 *Hypoxia*

Krebs-Henseleit solution was made hypoxic by replacing the normal $95\%O_2$:$5\%CO_2$ with a mixture of $95\%N_2$:$5\%CO_2$ for 1 hour prior to perfusion. On perfusion with the hypoxic solution cardiac contraction immediately decreased. After two minutes perfusion DT significantly fell from 6.8±0.9 to 1.1 ±0.4g ($p<0.05$). This degree of contraction was unable to trigger the rate meter.

Hypoxia also caused an immediate decrease in basal CPP. After 10 minutes perfusion with hypoxic perfusate, CPP fell from 88±2 to 68±8mmHg ($p<0.05$ compared to normoxic level). However, it did not affect the acidosis-induced coronary constriction. CPP still showed a transient decrease of 19±6mmHg on the perfusion with acidosis. Control coronary constriction in response to metabolic acidosis (pH 6.8), prior to hypoxia increased CPP from 88±2 to 158±12mmHg ($p<0.05$). Following 10 minutes of

hypoxia, acidosis increased CPP from 68±8mmHg increasing to 166±11mmHg ($p<0.05$ compared to hypoxic basal levels; see Figure 16). There was no significant difference between the normoxic and hypoxic acidotic constrictions.

3.11.2 Isopropyl palmitoyl carnitine

Isopropyl palmitoyl carnitine, P1Pi, is a compound that produces a long lasting coronary dilation, but the mechanism of action is not clear (Reeves *et al.* 1995). 10 minute perfusion with 1μM P1Pi had no effect on either cardiac contraction or rate. It did decrease basal CPP from 88±7 to 68±8mmHg ($p<0.05$). As was the case with coronary dilation produced by hypoxia, in the presence of P1Pi, acidosis (pH 6.8) still increased CPP. Figure R17 shows that the control response to metabolic acidosis (pH 6.8), increased CPP from 88±7 to 152±7mmHg, while in the presence of P1Pi from 68±8mmHg to 130±12mmHg (both $p<0.05$ compared to their respective controls).

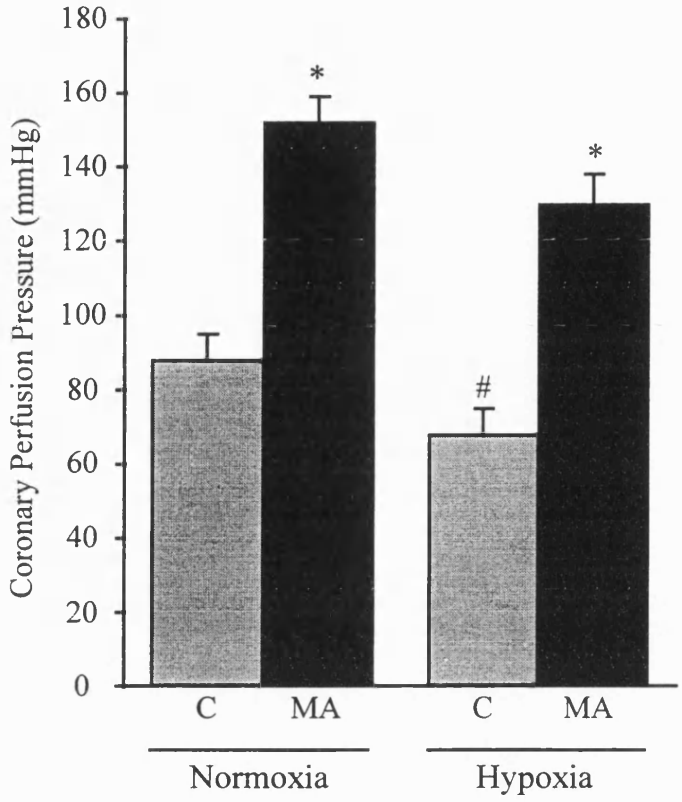


Figure R16: Cumulative histograms showing the effects of hypoxia on control CPP (C) and the CPP response to 5 minute application of metabolic acidosis (MA; pH 6.8) under normoxic and hypoxic conditions in the isolated rat heart.

Data are mean ± s.e.m., n=4. * $p<0.05$ compared to respective control (pH 7.4)

# $p<0.05$ compared to normoxic basal

It can be seen that perfusion with hypoxic buffer dilated basal CPP but did not affect CPP in the presence of metabolic acidosis.

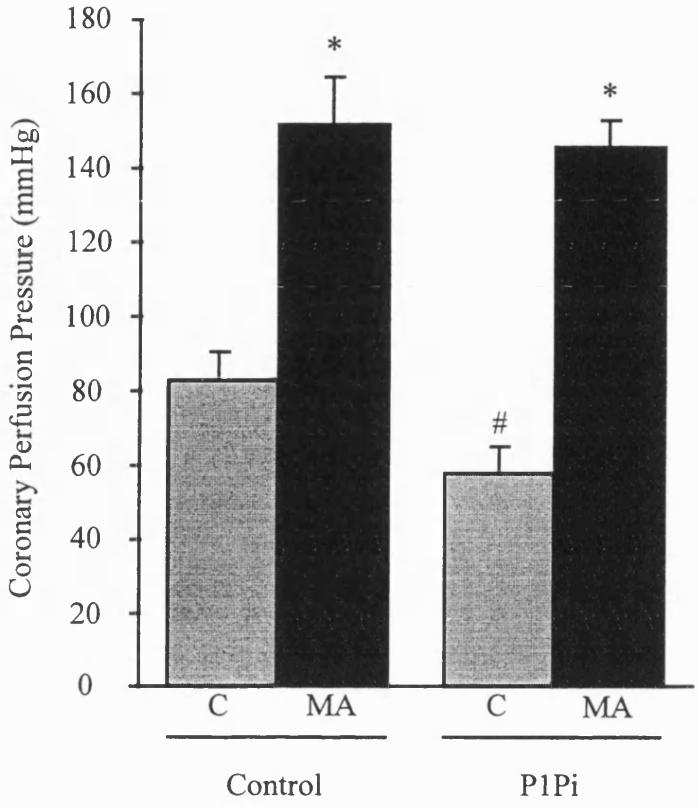


Figure R17: Histograms showing the effects of P1Pi (1μM) on control CPP (C) and CPP in response to and metabolic acidosis (MA; pH6.8) in the isolated rat heart.

Data are mean ± s.e.m., n=4. * $p<0.05$ compared to respective control (pH 7.4)

# $p<0.05$ compared to control levels (in the absence of P1Pi)..

Similar to hypoxia data, P1Pi decreased basal CPP but was ineffective against metabolic acidosis-induce dcoronary constriction.

3.12 *Effect Of Nifedipine On The Coronary Responses To Endothelin-1 And BAY K 8644*

The previous section presented evidence that the attenuation of the CPP response to acidosis produced by nifedipine was not due solely to its vasodilator properties since reduction in CPP by decreasing flow rate, hypoxia and P1Pi did not affect the acidosis-induced constrictions. Another possibility for the nifedipine results is that the compound is affecting the viability of the vascular smooth muscle non specifically. To address this, the ability of coronary smooth muscle to contract was examined using by administering bolus doses of the Ca^{2+} channel agonist BAY K 8644 or endothelin-1 (ET-1) in the presence of nifedipine

ET-1 causes contraction of vascular smooth muscle by several mechanisms while BAY K 8644 is a selective Ca^{2+}channel agonist. Therefore, if nifedipine is having a selective effect it should only block BAY K 8644-induced changes in CPP.

In control experiments performed using Krebs-Henseleit solution at pH 7.4, 30pmoles BAYK8644 caused significant increases in CPP from 95±9 to 157±9mmHg ($p<0.05$) without affecting cardiac contraction or rate. Responses returned to control levels within 1 minute. The same dose of endothelin-1 caused a more protracted constriction from 103±7 to 168±9mmHg ($p<0.05$). In the presence of nifedipine (100nM) the effects were significantly altered. The same bolus of BAYK8644 (30pmoles) caused no increase in CPP, while endothelin-1 (30pmoles) increased CPP, although reduced compared to control (see Figure R17).

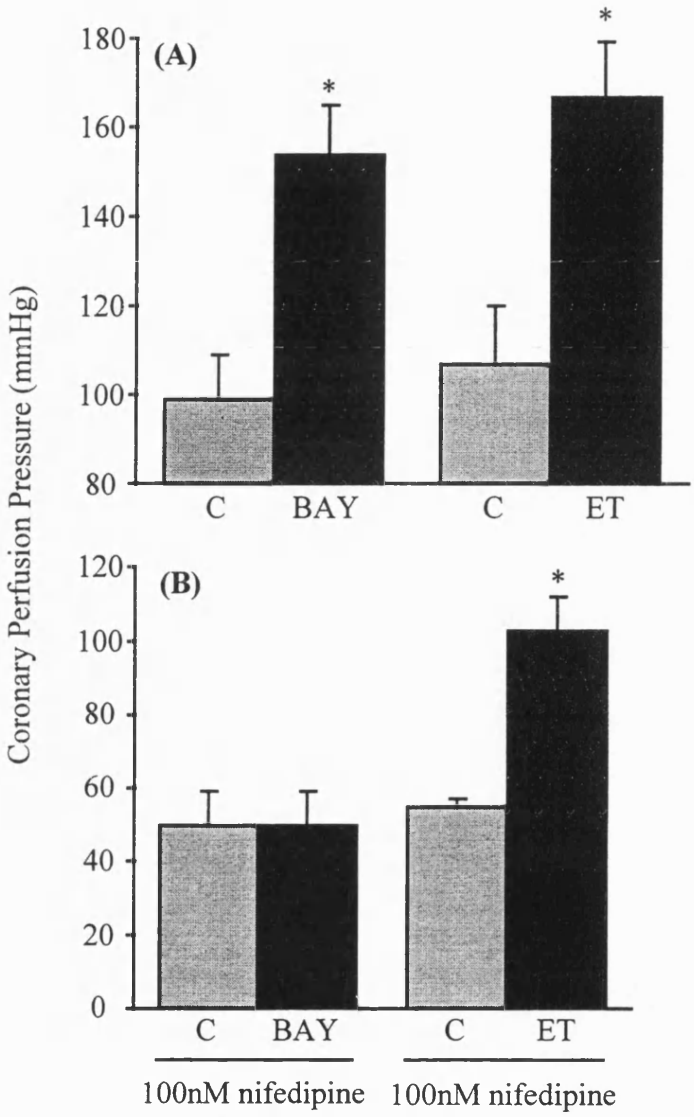


Figure R17: Cumulative histograms showing the effects nifedipine (100nM) on CPP responses to BAY K 8644 (30pmoles; BAY) and endothelin-1 (30pmoles; ET) in control (A) and (B) in the presence of nifedipine (100nM) in the isolated rat heart.

Data are mean±s.e.m., n=4.* $p < 0.05$ compared to control (C).

In the control situation, both BAY K 8644 and ET-1 were capable of causing significant constriction. In the presence of nifedipine, however, only ET-1 could elicit constriction.

3.13 *Effects Of Acidosis On Coronary Responses To Endothelin-1 And BAY K 8644*

The above results demonstrate that BAY K 8644 and endothelin-1 can constrict the vasculature of the isolated perfused rat heart. In addition, the data suggests that the BAY K 8644-induced constriction was entirely reliant on L-type Ca^{2+} channels, whereas endothelin-1 could cause constriction independent of these channels. Considering previous data demonstrating that acidosis-induced constrictions were dependent on the L-type Ca^{2+} channel, it was of interest to investigate whether these two compounds could cause constriction in the presence of acidosis and raised CPP.

When applied, metabolic acidosis increased CPP to 178±9 and 170±5mmHg in the experiments concerned. Figure R18 shows the data from such experiments. It can be seen that 30pmoles BAY K 8644 could not significantly increase CPP in the presence of metabolic acidosis. Endothelin-1 (30pmoles), however, was still capable of inducing significant additional constriction.

These results suggest that the nifedipine-induced attenuation of the coronary constriction observed with acidosis is not due to nifedipine affecting the viability of the vascular smooth muscle or a non-specific physiological effect. Furthermore, the inability of the Ca^{2+} channel agonist BAY K 8644 to elicit further constriction in the presence of metabolic acidosis could suggest that acidosis activated L type Ca^{2+} channels maximally. However, coronary constrictions observed with ET-1 in the presence of acidosis suggests that the vascular smooth muscle was capable of further constriction independent of L-type Ca^{2+} channels.

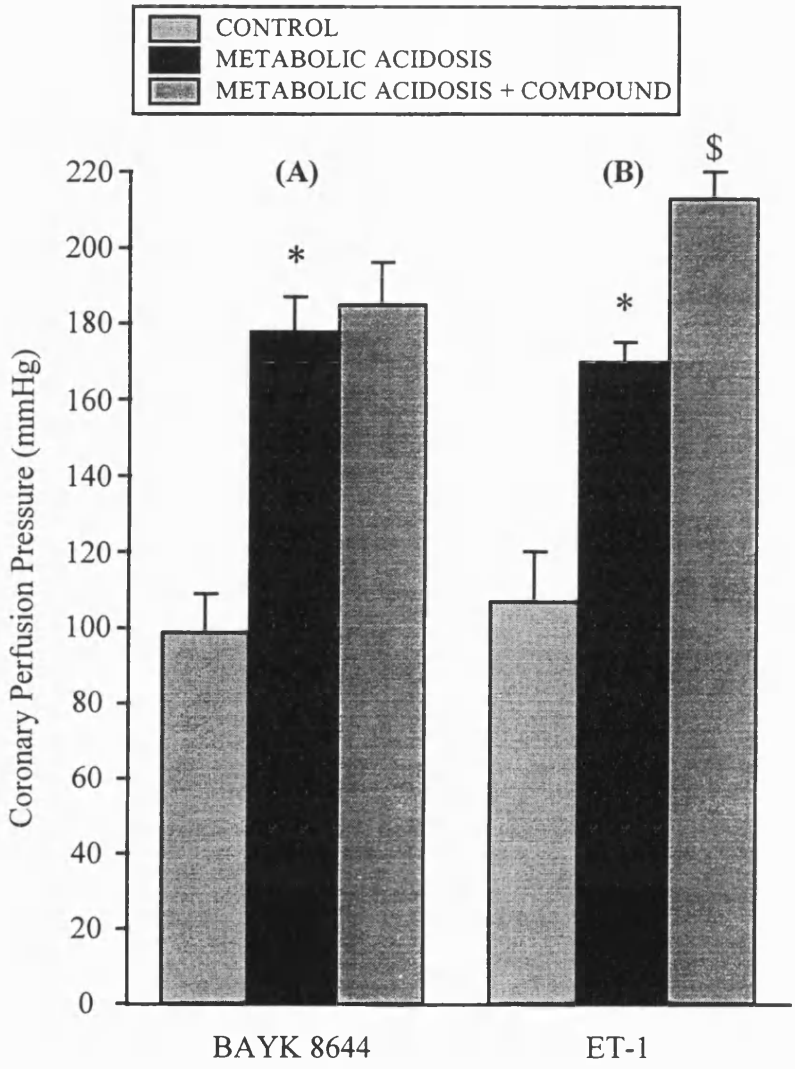


Figure R18: Cumulative histograms showing effects of BAYK8644 (A) and (B) endothelin-1 (both 30pmoles) applied in the presence of metabolic acidosis (pH 6.8) in the isolated rat heart.

Data mean ± s.e.m., n=5.* $p<0.05$ compared to basal level

$ $p<0.05$ compared to acidotic level

These data demonstrate that BAY K 8644, which had previously been shown to constrict in a nifedipine-sensitive manner, could not increase CPP further in the presence of metabolic acidosis. ET-1 was capable of further constriction.

3.14 *Effects Of K^+ Channel Blockers On The Isolated Perfused Rat Heart*

In view of the fact that K^+ channels play an integral role in the control of the membrane potential and hence the activation of L-type Ca^{2+} channels, the effects of a variety of K^+ channel blockers on the isolated rat heart was studied. The pivotal role of K^+ channels in the regulation of smooth muscle tone is now well established (Nelson & Quayle 1995). Extensive investigations have demonstrated the role played by voltage-sensitive K^+ channels in the control of membrane potential and, hence, tone of coronary arteries (Daut *et al.* 1994; O'Rourke 1996). In addition, K^+ channels have now been recognized as a target for endogenous vasodilators (Quayle & Nelson 1994).

A range of K^+ channel blockers; 4-aminopyridine (4-AP), glibenclamide (glib), tetrabutylammonium (TBA), tetraethylammonium (TEA) and penitrem A were perfused and their effects on coronary perfusion pressure noted. CPP reversibly increased in a concentration-dependent manner in response to the perfusion of 4-AP, penitrem A, TBA and TEA. Although it did not achieve statistical significance, glib also tended to increase CPP. The concentration response curves for both glib and penitrem A were stopped at their respective concentrations due to their solvent dilution factor increasing over 1:1000. These data are presented in figure R19. All of these constrictions were sensitive to nifedipine (100nM). Unlike CPP, which generally increased in response to the K^+ channel blockers, DT and HR showed concentration-dependent decreases in response to these agents. The only exception to this being the lack of effect of glib and penitrem A on HR. (see figure R20 and R21).

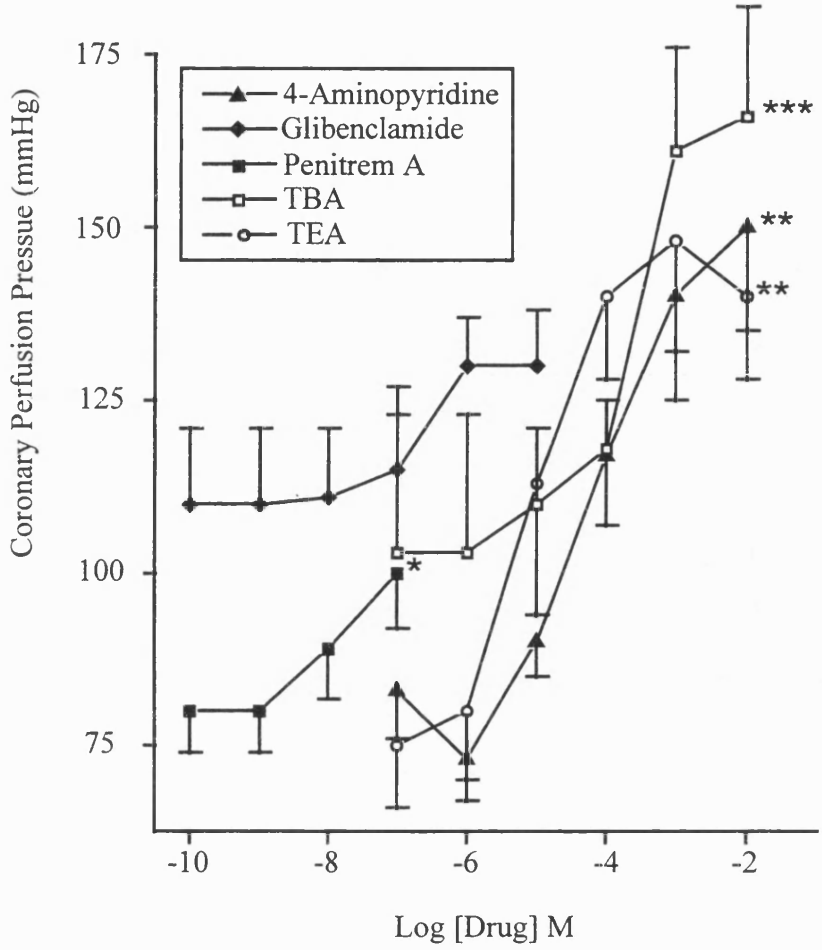


Figure R19: Concentration-response curves showing the effects various K^+ channel blockers on coronary perfusion pressure in the isolated perfused rat heart.

Data are mean ± s.e.m., n=5.

* $p<0.05$ compared to basal; ** $p<0.02$ compared to basal; *** $p<0.001$ compared to basal

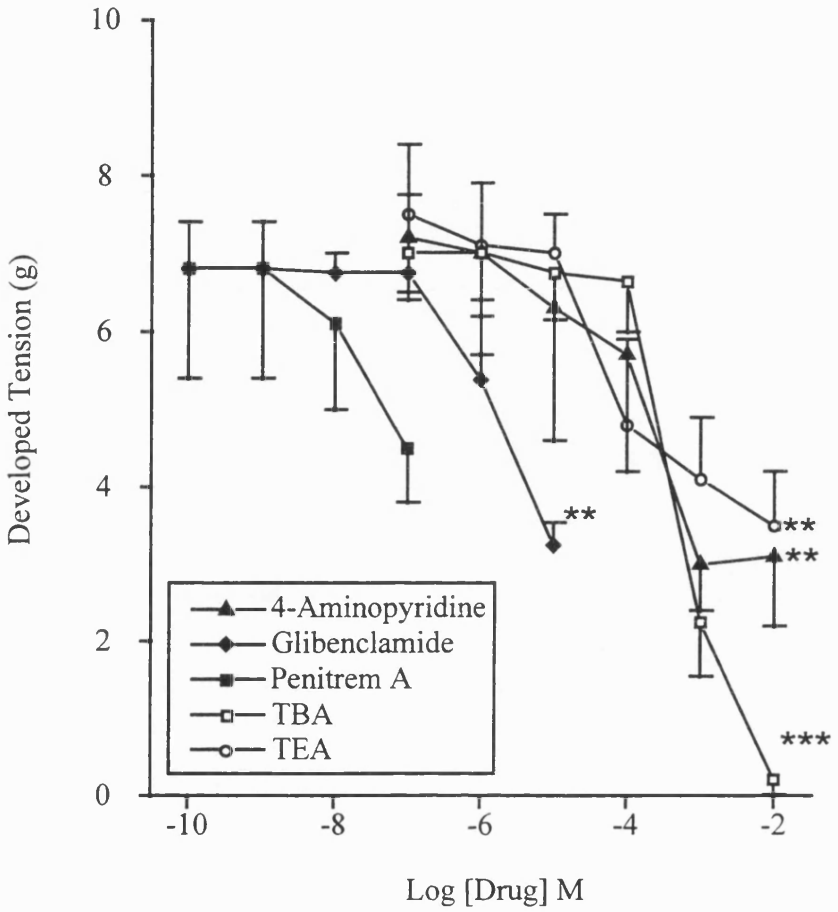


Figure R20: Concentration-response curves demonstrating the effects of K^+ channel blockers on cardiac contraction in the isolated perfused rat heart.

Data are mean ± s.e.m., n=5.

* $p<0.05$ compared to basal

** $p<0.02$ compared to basal

*** $p<0.001$ compared to basal

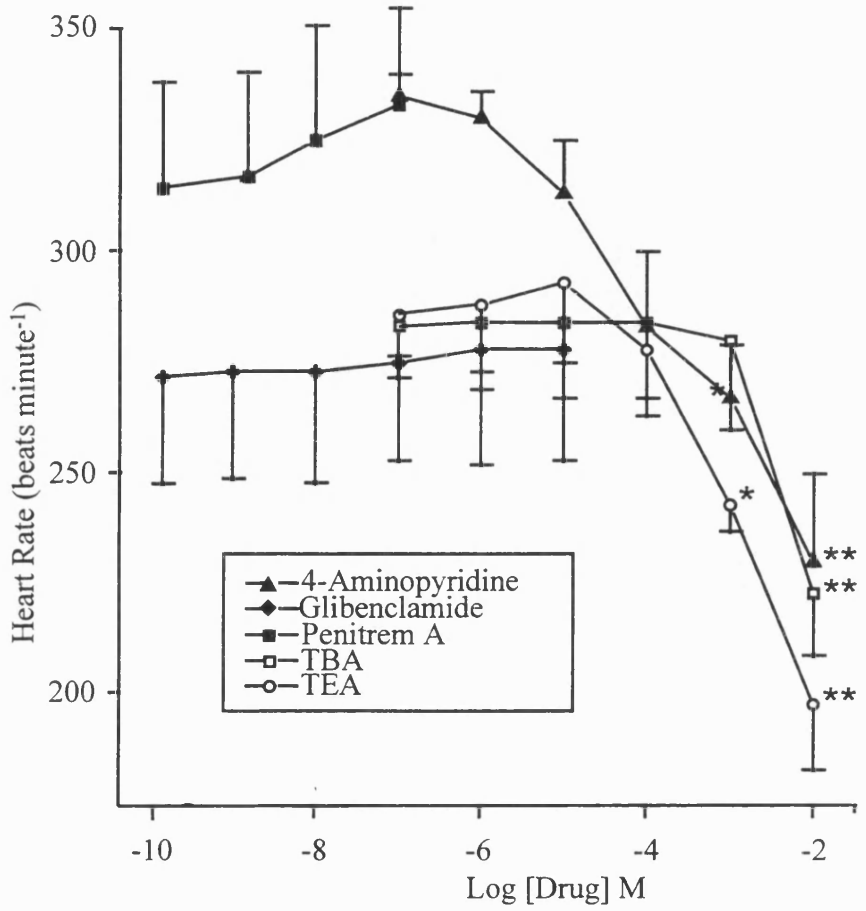


Figure R21: Concentration-response curves demonstrating the effects of K^+ channel blockers on heart rate in the isolated perfused rat heart.

Data are mean ± s.e.m., n=5.

* $p<0.05$ compared to basal

** $p<0.02$ compared to basal

*** $p<0.001$ compared to basal

4.0 DISCUSSION

The main focus of this thesis concerns the coronary vascular effects of acidosis produced using different protocols in the rat heart, and a comparison made with other vascular preparations in this and other species. As studies were carried out in whole hearts this also provided data concerning the effects of acidosis on cardiac contractility and heart rate. The data shows that the response to acidosis was dependent on how acidosis was induced, and also on the vascular tissue studied. In particular, the coronary constriction observed with acidosis in the isolated rat heart was dependent on extracellular Ca^{2+} entering through L-type Ca^{2+} channels.

4.1 Effects Of Experimental Manoeuvres Which Alter Intracellular pH On CPP

In the present experiments, comparisons have been made between intracellular pH acidified by two different techniques. In previous studies, both sodium butyrate, a weak acid, and NH_4Cl have been demonstrated to affect intracellular pH at a constant extracellular pH in vascular preparations (Taggart *et al.,* 1994). In response to butyrate (20mM), intracellular pH was rapidly (30 seconds to half-maximal response) and reversibly acidified by 0.18 pH unit. As discussed previously, NH_4Cl causes intracellular alkalinization on addition and acidification on wash-out. The study mentioned above measured these alterations as 0.23 and 0.15 pH unit respectively for NH_4Cl (20mM). Similar observations have been made in cardiac preparations (Bountra & Vaughan-Jones 1989).

The methods described above cause intracellular acidosis whilst maintaining extracellular pH. The majority of the present study, however, used changes in both extra- and intracellular pH. The proportion to which pH changes are transferred to the intracellular space is dependent on the degree of extracellular pH variation and cell type. Changes in extracellular pH have been demonstrated to be rapidly transferred into changes in intracellular pH in both cardiac (Bountra & Vaughan-Jones 1989; Nakanishi *et al.,* 1990), and vascular preparations (Austin & Wray 1993a). The ratio of intracellular:extracellular pH changes are, however, variable. Austin & Wray (1993a) reported a ratio of 0.73 pH unit in rat mesenteric strips. This figure is reduced in cardiac myocytes to about 0.4 pH units in response to extracellular pH 6.9. In agreement with this are results from experiments using both cardiac and coronary myocytes isolated from the same rat heart. In this study, Ramsey *et. al.,* (1994) altered extracellular pH from pH 7.4 to 6.9 with HCl. From a resting intracellular pH of 6.97±0.03 and 6.95±0.04 in cardiac and coronary myocytes, acidosis (pH 6.9) decreased this figure by 0.41±0.01 and 0.27±0.05 respectively.

4.2 Experimental Buffer Solutions

In the studies carried out in this study, extracellular acidosis was achieved by three different techniques. Namely, reduction of perfusate [HCO_3^-], increases in pCO_2, or using HEPES buffered solution to which HCl was added. However, the majority of experiments performed have used metabolic acidosis, *i.e.* reduction in [HCO_3^-]. All of these techniques have been widely used in the past as experimental tools to study the effects of acidosis on various preparations. The relationship obtained between perfusate pH and [HCO_3^-] (figure R1) is in agreement with other studies. Using 24mM [HCO_3^-]

and pCO_2 of 36mmHg, perfusate pH was 7.4. On reduction of [HCO_3^-] to 8, 3, and 1 mM, pH was 6.9, 6.5 and 6.0 respectively (Kagiyama *et al.,* 1982). Similar relationships from different groups were obtained (Veenstra *et al.,* 1987; Watters *et al.,* 1987; Nakanishi *et al.,* 1990).

Like the dependence of pH on [HCO_3^-], the quantity of CO_2, expressed as pCO_2 mmHg, required to acidify Krebs-Henseleit solution in this study is also similar to other studies. Bountra & Vaughan-Jones (1989) using 24mM [HCO_3^-] increased pCO_2 from 36 to 288mmHg to give perfusate pH 6.5 from pH 7.4. In a separate study, increasing the percentage of CO_2 from 5 to 20%, with 24mM [HCO_3^-], caused a decrease in 0.6pH units from 7.4 (Nakanishi *et al.,* 1990).

It can be clearly seen from the results that the sustained negative effects of acidosis on the isolated perfused rat heart are independent of buffer composition. Previous work has shown that alterations in [HCO_3^-] of experimental buffers alter the amount of free Ca^{2+} in the buffer (Fry & Poole-Wilson 1981). Although using this procedure to cause metabolic acidosis (pH 6.1-7.4) could increase the free Ca^{2+}, no such increase was detected using a Ca^{2+} sensitive electrode. Furthermore, the results obtained using HEPES-buffered Tyrode where pH is set with acid/alkali suggest that it is the acidosis produced by the decrease in [HCO_3^-] and not the potential increase in free Ca^{2+} that is responsible for the sustained coronary constriction that is seen.

4.3 *Bearing On pH Regulatory Mechanisms ?*

When different buffers were used at the same extracellular pH, these clearly had effects on the basal parameters being measured in the isolated rat heart. The practice of using non HCO_3^-/CO_2 buffers, HEPES buffered Tyrode solution, to eradicate any contribution of HCO_3^- -dependent mechanisms to intracellular pH regulation is common-place. However, if coronary perfusion pressure is used as an index of intracellular pH, replacement of HCO_3^-/CO_2 buffered Krebs-Henseleit with HEPES buffered Tyrode (both pH 7.4) solution caused a transient dilation recovering to control levels within 5 minutes (see page 79). Studies using pH-sensitive dyes have shown that changing from Krebs-Henseleit to HEPES buffered Tyrode solution causes intracellular alkalosis due to the rapid diffusion of CO_2 out of the cell (Vandenberg *et al.* 1996). This dilation, if it is mediated by intracellular alkalinization, correlates well with results from NH_4Cl pre-pulse experiments. Application of NH_4Cl, which leads to intracellular alkalinization, also induced a dilation (see figure R8).

Results obtained with HCO_3^- containing and HCO_3^- free buffers also show that the acidosis-induced coronary vasoconstriction seen in the isolated rat heart is independent of HCO_3^--dependent intracellular pH regulatory mechanisms as acidosis increased CPP in HCO_3^- containing and HCO_3^- free conditions. There are two other pH regulatory mechanisms which are independent of HCO_3^- which could possibly be affected by pH changes. The Na^+-H^+ exchanger usually removes intracellular protons in exchange for extracellular Na^+. This mechanism is unlikely to be responsible for the experimental observations since it will be working against a concentration gradient under conditions of extracellular acidosis. More conclusive evidence for the non-involvement of the

exchanger in the responses would be obtained by carrying out ionic substitution experiments using Na^+-free solutions.

A further ion exchanger discovered recently in guinea pig ventricular myocytes could be involved in the acidosis-induced coronary constriction. The novel chloride-hydroxy exchanger has been shown to play an integral role in the transfer of extracellular pH signals into the intracellular environment (Sun *et al.,* 1996).

4.4 Experimental Observations

4.4.1 pH-Induced Changes In HR

The normal rhythm of the heart is dependent on the re-generation of a signal (action potential) and its subsequent transmission to all parts of the myocardium via specialised communication routes *e.g.* Bundles of His (the normal route for transmission between atria and ventricles), the Purkinje fibre network and cell-to-cell communication. Acidosis has previously been demonstrated to have significant effects on both the action potential characteristics and its transmission throughout the heart.

Extracellular acidosis (pH 6.8) produced by all three techniques significantly decreased heart rate in both rat and guinea pigs. Cardiac action potentials are generated by the opening and closing of specific ion channels located in the plasma membrane of cardiac myocytes. The upstroke of the action potential, in ventricular muscle, is dependent on an influx of Na^+ through their channels. Whole-cell patch clamp experiments performed on neonatal rat ventricular muscle cells exposed to extracellular acidosis (pH 7.4-6.4)

revealed that a reduction in extracellular pH reduced peak current while intracellular pH altered the channel kinetics. Together, these effects reduced the channel activity (Watson & Gold 1995). This would result in a slowing in the maximum rate of rise of the upstroke of the action potential and a possible reduction in rate.

A further facet of the action potential that acidosis could possibly affect is the plateau. In experiments performed on guinea pig and rabbit papillary muscle, respiratory acidosis (pH 6.8) depressed the plateau phase of the action potential while prolonging its duration by approximately 10% (Fry & Poole-Wilson 1981). Acidosis (reduction in $[HCO_3^-]$ from 24 to 2.5mM) to a level of pH 6.3 from 7.4 had no effect on action potential duration, but did decrease the maximum rate of depolarisation and amplitude of the action potential (Campbell *et al.* 1991).

Acidosis has also been demonstrated to decrease the propagation of cardiac action potentials at the gap junctions. Using internal longitudinal resistance as an index of conductivity, acidosis produced by NH_4Cl pre-pulse increased resistance by 30%. A similar figure being obtained by increasing pCO_2 (Reber & Weingart 1982). Similar observations were obtained using Lucifer yellow transfer between adjacent cultured rat neonatal ventricular cells. The dye was transferred between cells via the gap junctions, this was reduced by 90% in acidotic conditions (Burt 1987). White *et al.,* (1990) used rat hearts to demonstrate that acidosis (pH 6.1 achieved by increasing CO_2) virtually abolished gap junction conductance.

Shaw & Rudy (1997) recently demonstrated that there is evidence against the role of acidosis in the observed negative chronotropic response. In their study, extracellular K^+ concentration is the major determinant of propagation velocity during acute ischaemia, with acidosis-induced reductions in conduction velocity only being observed under elevated K^+ levels

Although no measurements of action potential characteristics or conduction velocities were made, the majority of information implicates acidosis in the negative chronotropic effect described above.

4.4.2 pH-Induced Changes In DT

The results obtained in the course of this study correlate well with the vast amount of literature documenting the negative inotropic effect of acidosis on cardiac contraction (see introduction for explanation). Experiments performed using rat and guinea pig cardiac tissue demonstrated that all types of extracellular acidosis used caused a reversible decrease in cardiac contraction. Mechanisms of action for this inhibition of contraction were extensively discussed in the introduction.

Interestingly, upon perfusion with metabolic acidotic solution (pH 6.8), a transient positive inotropic effect prior to the decrease in cardiac contractility was observed. However, using a similar pH obtained with hypercapnia, there was no transient positive inotropic effect.

There are several possible explanations for this transient positive inotropic effect response. One possibility involves the manner in which extracellular pH signals affect intracellular pH. A study in rabbit ventricular myocytes demonstrated that metabolic, but not respiratory, extracellular acidosis resulted in a brief period of intracellular alkalosis immediately after altering extracellular pH (Nakanishi *et al.,* 1990). As alkalosis increases the sensitivity of the contractile proteins to Ca^{2+} this could underlie the transient inotropic action. A further possible explanation for the transient positive inotropic effect is acidosis increasing intracellular Ca^{2+}, prior to the inhibitory effects of protons on contractility. Protons are known to compete with Ca^{2+} for a common buffering site inside cells (Helzmann & Hunziker 1991), and have been shown to increase Ca^{2+} levels in a number of cell types. These include cardiac myocytes (Orchard and Kentish 1990), aortic vascular smooth muscle cells (Batlle *et al.* 1993), the human colon carcinoma cell line, HT_{29} cells (Nitschke *et al.,* 1996) and rat inner medullary collecting duct cells (Slotki *et al.,* 1993). Lastly, Na^{+}-Ca^{2+} exchange is one of the major routes for Ca^{2+} efflux from myocytes. Acidosis could possibly slow the exchanger, resulting in an increase in cytosolic Ca^{2+} levels, and hence contraction (Terracciano & Macleod 1994).

All the studies mentioned above, regardless of cell type, agree that intracellular acidosis increases free cytosolic Ca^{2+} levels. This could result in a positive inotropic effect, immediately before the inhibitory effects of the protons become apparent. The increase in Ca^{2+} also occurs under conditions of intracellular alkalosis (Batlle *et al.* 1993), possibly incorporating the scheme outlined above.

4.5 VASCULAR PREPARATIONS

4.5.1 Superior Rat mesenteric Bed

The previous section described the chronotropic and inotropic effects of acidosis in the isolated rat heart. In addition to experiments performed in the isolated heart preparation described above, the study also investigated the effects of extracellular and intracellular acidosis in the isolated perfused superior mesenteric bed of the rat. Acidosis did not affect basal tone in the superior mesenteric bed while the effects in pre-constricted preparations depended on the contractile agent used to increase tone and the method used to induce acidosis.

4.5.1.1 Dependence On Constrictor Agent

Alterations in extracellular and intracellular pH had no significant effect on K^+ constricted preparations. Depolarization induced by increasing extracellular K^+ will contract vascular preparations via activation of L-type Ca^{2+} channels and experiments performed with rat mesenteric and myometrial smooth muscle have demonstrated that contraction induced by K^+ is dependent on extracellular Ca^{2+} (Austin & Wray 1993b; Taggart *et al.*, 1996).

One possibility for the lack of effect in K^+-constricted preparations is that the activation of Ca^{2+} channels is supramaximal. Therefore, acidosis-induced block of these channels is not sufficient enough to overcome the effects of K^+ depolarization.

Taggart *et al.,* (1997) also observed in rat uterine smooth muscle that depolarization with K^+ (40mM) resulted in increased intracellular Ca^{2+} and maintained force production. A similar relationship between K^+ depolarization and intracellular Ca^{2+} was found in rabbit mesenteric artery (Nakanishi *et al.,* 1996).

K^+ depolarization of vascular smooth muscle is known as electromechanical coupling. A further mechanism used to increase mesenteric vascular tone was agonist-induced contraction, termed pharmacomechanical coupling. In the present study phenylephrine was used for this purpose.

Phenylephrine acts via α_1 adrenoceptors to activate PLC via a G-protein to generate IP_3 and DAG (Berridge 1993). IP_3 has been shown to mobilize intracellular Ca^{2+} in rat portal vein cells in a heparin and caffeine sensitive manner (Loirand *et al.,* 1994). PKC, the target for DAG, has been implicated in the enhancement of myofilament sensitivity with respect to Ca^{2+} in vascular smooth muscle (Rokolya *et al.,* 1994).

Jensen *et al.,* (1992) demonstrated that, in rat mesenteric arteries, noradrenaline and K^+ caused similar increases in intracellular Ca^{2+} levels. In the presence of 125mM K^+, noradrenaline increased force with no increase in Ca^{2+} levels. This means that tension/$[Ca^{2+}]$ ratio can be altered with certain agonists. Furthermore, in response to α-adrenoceptor activation, this ratio is increased. Thus, agonists can increase the efficacy of intracellular Ca^{2+}. This heterogeneity of response with respect to contractile agent has also been previously reported (McKinnon *et al.,* 1996; Nakanishi *et al.,* 1996).

4.5.1.2 K^+-Depolarized Preparations

The experiments performed using K^+ depolarized preparations, it was found that these preparations were found to be generally unaffected by alterations in both extracellular and intracellular acidosis (see figures R7 & R9). If the relationship between K^+ depolarization and intracellular Ca^{2+}, plus myosin light chain phosphorylation, exists in rat mesenteric vascular smooth muscle (Taggart *et al.,* 1997), it could indicate that acidosis does not alter any steps involved in excitation-contraction concerned with K^+-induced depolarization provided that the effects K^+ are not supramaximal. It could be argued that the concentration of K^+ used to depolarize the tissues was too high, possibly saturating the contractile machinery and not allowing any acidosis-induced manipulation of this relationship to be observed. However, examination of reports which used K^+ depolarization to constrict mesenteric preparations show that the concentration used here to be in a similar concentration range.

Interestingly, Austin & Wray (1993b) performed a study using strips of mesenteric artery obtained from vessels of radii 150μM. In this study, 60mM K^+ was used to constrict preparations with extracellular acidosis (pH 6.9; achieved by addition of HCl) reversibly dilating the preparations. This dilation, in a later study, was shown to associated with a decrease in cytosolic Ca^{2+} (Austin & Wray 1995). Furthermore, this study also suggested that this dilation can be accounted for by alterations in Ca^{2+} entry since increases in extracellular Ca^{2+} could reverse the dilation.

This major difference in response between the study of Austin & Wray (1993b) and the work in this study is the vessel preparation. Here, the entire mesenteric bed is perfused with presumably the different diameter vessels contributing to the observations. In the study of Austin & Wray (1993b), only one diameter of vessel is used (radii 150μM). It could be that the response to acidosis is dependent on vessel calibre, in which case the preparation used here could be considered more physiological.

4.5.1.3. Agonist-Induced Constricted Preparations

Responses to acidosis observed in the rat superior mesenteric bed were not only dependent on the constrictor agent used, but also on the method used to impose acidosis. Phenylephrine-constricted preparations dilated in response to metabolic acidosis, but constricted in response to respiratory acidosis, butyrate- and NH_4Cl-induced pH changes (see figures R7 & R9).

Experiments performed with phenylephrine (10μM) raised MPP to a similar level as that seen with K^+-depolarization. So, it is reasonable to assume that the differences between the two constrictor agents are not pressure related. As described in the introduction, the relationship between Ca^{2+} and tension is more complex for agonist-induced, compared to K^+ constricted preparations. The multiplicity of contractile pathways with agonist-induced contraction means that there are potentially more mechanisms for the observed acidosis-induced changes in MPP.

In response to metabolic acidosis, MPP reversibly decreased. There are many possible mechanisms that could account for the relaxation of phenylephrine-constricted preparations which include inhibition of any of the steps involved in calmodulin-dependent phosphorylation of MLCK, increased NO production (Matthews *et al.,* 1992) or activation of ATP-sensitive K^+ channels which could in turn block L-type Ca^{2+} channels (Ishizaka & Kuo 1996).

In phenylephrine constricted preparations, respiratory acidosis (pH 6.8) caused a further constriction. This constriction was not well sustained, although MPP was still above resting levels at the end of the 5 minute experimental period. CO_2-induced constriction of mesenteric vessels has been previously reported (Matthews *et al.,* 1992). This study used vessels of 200-350μM internal diameter mounted in a myograph, contracted with 5μM noradrenaline, and examined the effects of 5, 10 ,and 20% CO_2 (pH 7.1, 6.9 and 6.7 respectively). In response to increasing percentages of CO_2 tension increased in a concentration-dependent manner. This constriction was also transient, after 5 minutes in the presence of the increased CO_2, tension reversed back to normal values. This reversal was later demonstrated to be due to increased NO production since it was not seen in endothelial denuded vessels or in the presence of L-NAME (Carr *et al.,* 1993).

Austin *et al.,* (1996) measured intracellular Ca^{2+}, pH and tension in rat mesenteric vessels. They showed that alterations in extracellular pH (whether alkalosis or acidosis) altered intracellular pH, which in turn altered intracellular Ca^{2+} which lead to alterations in tension. This direct effect on Ca^{2+} leading to changes in tension is in contrast to another study where pH-induced alterations in contractile apparatus sensitivity was observed (Crichton *et al.,* 1993). Further work is needed to investigate whether

alterations in Ca^{2+} influx or contractile apparatus sensitivity underlies the differences observed using the superior mesenteric bed in the work here.

4.6 *Coronary Vascular Effects Of Acidosis*

The primary aim of this study was to study the effects of acidosis on vascular smooth muscle contractility. In order to achieve this, rat aorta and superior mesenteric beds have been used. But principally, the effects of acidosis have been concerned with the coronary circulation of the rat and, to a lesser extent, the guinea pig and pig.

The Langendorff-perfused whole heart not only allows the investigator to observe cardiac contraction and heart rate (these effects have already been discussed), but also the contraction of the vascular smooth muscle cells of the coronary circulation. Results with all three types of acidosis employed in this thesis show that the coronary vasculature of the isolated rat heart undergoes a transient dilation followed by a sustained constriction in response to acidosis, whereas the coronary circulation of the guinea pig dilates.

In porcine coronary artery rings acidosis did not affect basal tension. Porcine coronary artery rings did not constrict to phenylephrine (>10μM). This has previously been reported (Morgan & Morgan 1984). However, porcine coronary artery rings were responsive to K^+-induced depolarisation. Following K^+ depolarization acidosis produced a relaxation in the pig coronary artery preparation *i.e.* a similar effect to that seen in guinea pig coronary vessels.

Furukawa *et al.*, (1996) reported extracellular acidosis-induced constrictions in a non-coronary vessel preparation, the isolated rat aortic spiral preparations. This constriction was also demonstrated to be sensitive to removal of extracellular Ca^{2+}. However, when a similar protocol was repeated in the laboratory here, extracellular acidosis-induced constrictions were not observed. These results show both species and regional differences in the effects of acidosis on blood vessels.

4.6.1 Separation Of Coronary Vascular Effects Of Acidosis From The Myocardial Effects

Results obtained during the course of the study showed that acidosis (pH 6.8) caused the expected decrease in myocardial contractility and a concurrent increase in CPP which was not expected. Because of the intimate relationship between coronary and cardiac myocytes (Daut *et al.*, 1994), due to release of vasoactive substances from cardiac muscle, and the direct physical effects of cardiac contraction on coronary vascular smooth muscle, it was important to separate the myocardial and vascular effects of acidosis.

The experiments performed to answer the question asked above used electrical pacing and K^+ arrested hearts. Increasing the rate of stimulation (up to 16Hz) resulted in a decrease in cardiac contractility (Borzak *et al.*, 1991), while perfusion with 15mM total extracellular K^+, cardiac contractility immediately ceased. This experimental protocol of using increased extracellular K^+ to arrest cardiac contraction has been previously used (Cyrys & Daut 1994). Coronary constrictions obtained with metabolic acidosis (pH 6.8) under these conditions were not significantly different to control constrictions prior to

the hyperkalemic manoeuvres. This would indicate that acidosis-induced constrictions are not secondary to the myocardial depressant effects of acidosis and do not require an active myocardium.

Further indications that the acidosis-induced coronary constriction occurs independently of the myocardial depressant effects are seen with the experiments concerned with hypoxia. As in the hyperkalemia and pacing experiments, DT was depressed under the hypoxic conditions (see page 119) but metabolic acidosis still elicited coronary constriction which was not significantly different to the control response.

These results indicate that the coronary constriction in response to acidosis which was seen in the rat heart is independent of the cardiac depressant effects of acidosis and are a direct effect of acidosis on the vasculature, or at least, some cell type other than the cardiac myocyte.

4.6.2 Transient Coronary Dilation In Response To Acidosis: possible mechanisms

On the application of metabolic acidosis, there were alterations in CPP and DT. Immediately on perfusion with metabolic acidosis there was a transient positive inotropic effect prior to the sustained negative effect. Interestingly, it would appear that these changes are not due to the changes in pH *per se.* Rather, the method employed to assert acidosis was responsible. Application of metabolic acidosis (pH 6.8) caused the transient increase and decrease in DT and CPP respectively. However, on application of respiratory acidosis or HEPES-buffered Tyrode (both pH 6.8), there was no transient

change in CPP or DT only a sustained increase in CPP and decrease in DT. Similar responses were observed using isolated guinea pig hearts.

One possible mechanism that could account for the transient dilation in coronary tone seen following the onset of acidosis is inhibition of L-type Ca^{2+} channels as would be expected on the basis of single cell patch clamp studies (Klöckner and Isenberg 1994a &b). A further possibility is an increased production/release of an endogenous vasodilator *e.g.* adenosine or NO. Matthews *et al.* (1992) described acidosis-induced dilation of rat mesenteric vessels which is dependent on the presence of the endothelium and attenuated by L-NAME. Further processes may include acidosis-induced dilation (porcine coronary arteries) via ATP-sensitive channels (Ishizaka & Kuo 1996). This latter possibility was not investigated further in this thesis. The increase in NO production can be discounted since the transient dilation was still observed in the presence of NO-ARG. In addition, NO production via cNOS is pH-dependent, with acidosis decreasing NO production (Fleming *et al.* 1994). Likewise, the transient dilation observed with metabolic acidosis is independent of increased adenosine production since the non-specific adenosine antagonist, 8-PT, did not attenuate the dilation. Furthermore, cyclo-oxygenase products, such as prostacyclin, can be discounted since the dilation also occurred in the presence of indomethacin. This is not to say that cardiac contractility could be producing a vasodilator metabolite other than adenosine, cyclo-oxygenase products or NO.

Interestingly, the transient dilation did not occur when myocardial contractility had been initially depressed by hyperkalemia, hypoxia or pacing. Under these conditions acidosis did not produce a transient increase in DT. Therefore, the dilation could be secondary to

the increase in DT releasing a metabolic dilator substance or via the garden hose effect A further line of indirect evidence for the link between contractility and coronary tone is seen following the application of NH_4Cl to the isolated rat heart. In response to the intracellular alkalosis observed on the application of NH_4Cl, there is an increase in DT and a decrease in CPP.

There is a direct physical link between cardiac contraction and coronary flow known as the "garden hose" effect. This term describes the relationship between cardiac contraction and coronary flow. It has been postulated to play a causal role in ischaemic-induced contractile dysfunction (Figuerdo *et al.*, 1992). Within this, a decrease in intravascular pressure leads to a decrease in cardiac contraction. The authors of this study proposed that the relationship between Ca^{2+} and tension was responsible for the observations. Similarly, there has been an extensive study performed using Langendorff-perfused ferret hearts investigating the effects of differing coronary arterial pressures on cardiac contraction. Increases in coronary artery pressure, which correspond to increases in coronary flow rate (vasodilation), increased cardiac systolic Ca^{2+} levels and contraction. The authors proposed that this alteration in the levels of Ca^{2+} within the cardiac myocyte underlie the contractile changes (Kitakaze & Marban 1989). However, increasing the flow rate from 10 to 20 ml.minute^{-1} in this study did not increase cardiac contraction. However, decreasing the flow rate to 2.5 ml.minute^{-1} did decrease cardiac contraction. Increasing flow rate to 5 ml.minute^{-1} did reverse this effect, it is possible that the effect described by Kitakaze & Marban (1989) was overcome at flow rates greater than 5 ml.minute^{-1}.

The transient dilation was not observed when cardiac contraction was suppressed by hyperkalemia or pacing. This could indicate that the dilation occurred as a consequence of the brief positive inotropic effect. Or, that the brief positive inotropic effect occurred as a result of the brief dilation through a scheme outlined in the previous paragraph. However, further work is needed to finally conclude which transient effect is dependent on the other.

4.6.3 Acidosis-Induced Coronary Constriction In The Rat Heart

In response to acidosis (metabolic, respiratory or HEPES-buffered Tyrode; pH 6.8), there was a reversible negative effect on DT and HR in both the isolated rat and guinea pig hearts. However, there were different reproducible, reversible effects of acidosis on the coronary tone of the three species used. In response to acidosis, K^+ constricted porcine coronary artery relaxed and guinea pig CPP underwent a sustained decrease. However, rat CPP underwent a transient dilation before a sustained constriction. It could be argued that the transient dilation observed in the rat occurs through the same mechanism which is responsible for the sustained dilation in the guinea pig. If this is the case, then the mechanism responsible for the constriction in the coronary circulation of the rat could be missing from that of the guinea pig.

4.6.3.1 *Constant Flow And Constant Head Of Pressure In The Rat Heart*

Results obtained using constant flow and head of pressure were used to calculate values for coronary resistance under normal and acidotic conditions. Coronary resistance was used as an index of coronary tone in order to compare the effects of acidosis applied using constant flow and a constant head of pressure. By using a constant flow rate and forcing the solutions through the vessels, possible shear force could be activated leading to the release of a vasoactive substance (Shen *et al.*, 1992; Schwarz *et al,* 1994). There was no significant difference between values for vascular resistance obtained using the two experimental systems used *i.e.* in the rat acidosis increases coronary resistance. It could be concluded that using a constant flow rate does not influence any shear stress-induced release of vasoactive agents.

4.6.4 *Effects Of Intracellular Acidosis Only In The Rat Heart*

Application of NH_4Cl induced a relaxation of the coronary circulation which partially recovered in the continued presence of the compound. This relaxation induced by NH_4Cl has also been observed in rat mesenteric vessels (Matthews *et al.*, 1992). This study also measured intracellular pH and showed that the relaxation was associated with an intracellular alkalinization.

On removal of NH_4Cl, CPP significantly increased above control levels returning to these levels within 5 minutes. In mesenteric vessels, Matthews *et al.,* (1992) also observed this constriction. It was significantly reduced by the removal of extracellular Ca^{2+} and in the presence of the Ca^{2+} channel antagonist verapamil. This would indicate that Ca^{2+} entry through voltage sensitive Ca^{2+} channels is pivotal to the constriction observed under these conditions. This increase in intracellular Ca^{2+} in response to NH_4Cl washout has also been observed in canine and ferret pulmonary arterial smooth muscle (Krampetz & Rhoades 1991; Farrukh *et al.,* 1996).

In contrast to this, when acidosis was applied with butyrate which is reported to give a selective decrease in intracellular pH, this was shown to cause a dilation in the coronary circulation of the rat. Interestingly, the effects of the weak acid butyrate are presumed to be due to intracellular acidosis. However, recently it has been demonstrated in rat mesenteric vessels that lactate, another weak acid used to cause intracellular acidosis, can cause dilation independent of intracellular pH changes. This study also demonstrated that the significant effect of the contractile agent can affect the observations (McKinnon *et al.,* 1996). Rat mesenteric arteries constricted by K^+ (45mM) were unresponsive to application of lactate. In contrast lactate relaxed vessels constricted by noradrenaline (10μM). This is in agreement to the results obtained in the isolated rat heart in this study. Furthermore, when the pH changes were nullified, the lactate-induced dilation was still present. However, the competitive PKA inhibitor, R_p-cAMPS, was capable of reducing the lactate-induce dilation. This would implicate activation of adenylate cyclase and subsequent formation of cAMP in the response.

4.7 *Possible Mediators Of Acidosis-Induced Constriction In The Rat Heart*

There is a further cell type which, like the cardiac myocytes, has a similar intimate relationship with the coronary smooth muscle cells. These are the endothelial cells which form a continuous layer of cells lining all blood vessels. In addition to forming a physical barrier these cells are also capable of producing a series of vasoactive compounds *e.g.* NO, PGI_2, TxA_2 *etc.* (Wadsworth 1994). If there is a basal production of a dilator compound, *e.g.* NO or PGI_2, acidosis could possibly cause coronary constriction by inhibiting its production or action. Conversely, acidosis could increase the production/release of a constrictor agent *e.g.* TxA_2.

In the present study, the increase in CPP following addition of NO-ARG (100μM) for 15 minutes would suggest that the coronary circulation of the rat is under the influence of basal NO production. This concentration of the inhibitor has been demonstrated to be effective in other vascular preparations (Criddle *et al.,* 1994; Costa *et al.,* 1996; McKinnon *et al.,* 1996). However, in the presence of NO-ARG metabolic acidosis still elicited coronary constriction. As NO synthesis had already been inhibited by NO-ARG, this would suggest that the acidosis-induced coronary constriction is not due to inhibition of basal NO production.

Similarly, it is unlikely that acidosis is inhibiting the basal release of PGI_2 to cause constriction . This conclusion is reached because acidosis was capable of increasing CPP in the presence of indomethacin which would have already inhibited PGI_2 production. The fact that indomethacin did not affect basal tone suggests that cyclo-

oxygeanse products do not have a significant role to play in the regulation of basal coronary tone in the rat heart.

Franco-Cerada *et al.* (1994) have shown that low pH can release cyclo-oxygenase products from nerve endings in guinea pig hearts. This release was sensitive to incubation with indomethacin (10μM). The acidosis-induced constriction was shown to be independent of inhibition of vasodilator and vasoconstrictor cyclo-oxygenase products *e.g.* prostacyclin and TxA_2 as indomethacin (10μM), a concentration which is regularly used (McKinnon *et al.,* 1996) was shown to have no effect on the coronary constriction. Furthermore, the TxA_2 receptor antagonist and synthase inhibitor, ZD1542, was also shown to be ineffective at an effective concentration (Brownlie *et al.,* 1993).

One further mechanism which could be responsible for the acidosis-induced coronary constriction is inhibition of the release or actions of endogenous adenosine. There are reasons why this is unlikely to be the case. Firstly, in the presence of an effective inhibitory concentration of the adenosine antagonist 8-PT which blocked the dilation induced by bolus administration of adenosine increased basal CPP. This would indicate that adenosine does regulate basal coronary tone in the rat heart. However, the same concentration of 8-PT used, did not block the acidosis-induced increase in CPP.

Therefore, it can be concluded that the acidosis-induced coronary constriction was independent of inhibition of NO production, adenosine release and cyclo-oxygenase products especially TxA_2.

4.8 *Vasodilator Manoeuvres And The Effects On Acidosis-Induced Constriction In The Rat Heart*

It has been shown that nifedipine (1-100nM) significantly attenuated the acidosis-induced coronary constriction. In addition to this, nifedipine also significantly reduced basal CPP. It could be that this reduction in basal CPP was the mechanism through which the attenuation of the acidosis-induced constriction occurred. Hypoxia and P1Pi were used to decrease basal CPP to investigate whether this effect of nifedipine could be mimicked by other procedures which decrease CPP, and to test whether acidosis could still increase CPP in their presence.

Hypoxia, achieved by aerating the experimental solutions with $95\%O_2;5\%N_2$, was used to dilate the coronary vasculature. It has been demonstrated that hypoxic vasodilation occurred in K^+ arrested guinea pig hearts. Time of onset for the dilation was 15-30 seconds. The dilation was abolished in the presence of the sulphonylurea glibenclamide (2-5μM) (Daut *et al.* 1990; Cyrys & Daut 1994). This would strongly suggest that hypoxia stimulates the ATP-sensitive K^+ channel producing hyperpolarization of the smooth muscle cell. This hyperpolarization reduces the open probability of the L-type calcium channel, thus decreasing cytosolic free Ca^{2+} levels and hence a reduction in smooth muscle tone.

Further evidence for a hypoxic-induced, glibenclamide-sensitive dilation was gained from experiments using isolated smooth muscle cells from the porcine coronary artery. Perforated patch experiments revealed that hypoxia induced a current, whose reversal potential shifted with changes in extracellular K^+ levels as expected for a K^+ current.

The time of onset for the current was approximately 2 minutes and was inhibited again by glibenclamide (10μM) (Dart & Standen 1995). This study also highlighted the fact that the isolated cells are isolated and so cannot be influenced by endothelial factors. Such factors can be released from endothelial layer to either cause contraction or relaxation of smooth depending on the experimental conditions (Wadsworth 1994).

In the experiments performed here, hypoxia did dilate the coronary circulation of the rat heart, although the mechanism was not investigated further. Hypoxic-induced decreases in CPP did not attenuate the increase in CPP observed with acidosis (see figure R16).

4.8.1 P1Pi

P1Pi, the isopropyl ester of palmitoyl carnitine, has previously been demonstrated to be a vasodilator of rat coronary and mesenteric circulations although the mechanism of action is as yet unclear (Criddle *et al.* 1994). It was demonstrated that P1Pi caused dilation independent of cyclo-oxygenase products, ATP-sensitive K^+ channels or endothelium derived NO. This would indicate that it was possibly a direct effect on the vascular smooth muscle. Further evidence was provided that P1Pi had L-type Ca^{2+} channel blocking properties since it attenuated vasoconstrictions in response to cumulative additions of Ca^{2+} in K^+ depolarized mesenteric beds.

In this study, P1Pi dilated the coronary circulation of the rat. The mechanism of this is unclear although results with mesenteric beds would favour blockade of current through L-type Ca^{2+} channels. If this was the situation, P1Pi would have been thought to

attenuate the acidosis-induced coronary constriction. However, no effect was observed (see figure R17).

All the procedures utilised to dilate CPP (hypoxia, nifedipine, P1Pi and reduction in flow) all decreased flow by a different degree. Hypoxia and P1Pi were the least effective, while nifedipine dilation was only bettered by halving the flow rate. Since nifedipine-induced dilation was similar to the dilation induced by hypoxia and P1Pi, it would suggest that the attenuation of the acidosis-induced coronary constriction by nifedipine is not due to a physiological antagonism, but rather due to its Ca^{2+} channel antagonism properties.

4.9 Dependence On Extracellular Ca^{2+}

4.9.1 Basal CPP

There was an interesting difference between the effects observed under Ca^{2+} free conditions and the application of Ca^{2+} channel antagonists. On perfusion with Ca^{2+} free Krebs-Henseleit solution, cardiac contraction immediately ceased, as would be expected. Surprisingly, following an initial dilation, CPP remained at a level similar to the control values. This could imply that the residual Ca^{2+} concentration within the vascular smooth muscle cells is sufficient to maintain tone within the coronary circulation of the isolated rat heart. However, the decrease in CPP obtained with Ca^{2+} channel antagonists would suggest that the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels plays a pivotal role in the maintenance of basal tone.

This maintenance of CPP under nominally Ca^{2+}-free conditions contrasts with experiments performed with the Ca^{2+} channel blockers amlodipine, nifedipine and verapamil where application of these compounds inhibited Ca^{2+} entry and thus led to coronary dilation. These data would indicate that L-type Ca^{2+} channels do play a role in regulating basal coronary tone. These observations could indicate that either Ca^{2+} re-cycling or trace Ca^{2+} levels contained within the buffers could be responsible for the maintenance of tone in Ca^{2+}-free conditions. Alternatively, intracellular Ca^{2+} could be sufficient to maintain basal tone. This could be seen as an unlikely situation since it would mean that the Ca^{2+} channel antagonists would have an intracellular effect.

With Ca^{2+} channel antagonists there was a decrease in DT and CPP as expected. But the results with Ca^{2+}-free solution only DT decreased. The reason for this not clear but one possibility is that there could be re-cycling of intracellular Ca^{2+} *i.e.* when intracellular Ca^{2+} is extruded from the cell it immediately enters the cell again through voltage-sensitive Ca^{2+} channels. Since there was no attempt to buffer any Ca^{2+} which may be contained within the water used as the basis for the experimental buffers. Addition of a Ca^{2+} chelator, such as EGTA, to the Ca^{2+}-free solutions to buffer any trace levels of Ca^{2+} could elucidate the situation further. Since acidosis depressed cardiac contractility, the return to control levels of CPP could reflect a loss of vasodilator substance produced by the inactive myocardium.

In addition, the decrease in DT may cause a decrease in No and adenosine which would lead to an increase in CPP and oppose the effects of Ca^{2+}-free conditions.

4.10 *Potential Role Of L-type Ca^{2+} Channels In Acidosis-Induced Constriction*

In control studies after 5 minutes application of metabolic acidosis, CPP was significantly increased above control levels. Similar observations were observed with respiratory acidosis and HEPES buffered Tyrode solution-induced acidosis (both pH 6.8). The inability of acidosis (pH 6.8) to constrict the coronary circulation of the isolated rat heart in the absence of extracellular Ca^{2+} and in the presence of L-type Ca^{2+} channel blockers would indicate an implicit dependency on the entry of extracellular Ca^{2+} through such channels.

Since nifedipine reduced basal CPP, the inability of acidosis to constrict coronary vessels of the rat could be due to a non-specific effect of nifedipine. In order to investigate this possibility BAY K 8644 (the Ca^{2+} channel agonist) and ET-1 were used. Both of these compounds increased CPP under basal conditions. In the presence of nifedipine BAY K 8644 was ineffective. Similar results were obtained by Asano *et al.* (1987) using canine coronary arteries. Here, contractions to BAY K 8644 were competitively antagonized by nifedipine. This contrasts with the results obtained with the contractions obtained with ET-1 which were only partially blocked by nifedipine. Balligand & Godfraind (1994) using human coronary arteries and veins again demonstrated this partial antagonism, this time using nisoldipine, another di-hydropyridine. This shows that nifedipine is not simply preventing the acidosis-induced increase in CPP by acting as a physiological antagonist of vascular smooth muscle contraction.

Similar responses were observed when BAY K 8644 and ET-1 were applied in the presence of acidosis. In the presence of acidosis and increased CPP, the same bolus dose of BAY K 8644 could not cause further increase in CPP. In contrast, ET-1 could elicit further increases in CPP. Unless the inability of BAY K 8644 was pressure related or acidosis affected the binding of BAY K 8644 to its receptor, these results could suggest that acidosis had already activated L-type Ca^{2+} channels maximally. ET-1, however, which has multiple mechanisms to cause contraction increased CPP further. Indeed, the ET-1-induced increases in CPP in the presence of acidosis and nifedipine were similar, possibly indicating that acidosis had activated L-type Ca^{2+} channels.

This is further evidence that acidosis constricted the coronary circulation of the isolated rat heart via L-type Ca^{2+} channels

4.11 Potential Role Of Ca^{2+} And K^+ Channels In Acidosis-Induced Constriction

The observations that acidosis-induced increase in CPP is attenuated under nominally Ca^{2+}-free conditions, in addition to the attenuations seen with L-type Ca^{2+} channel blockers, would implicate voltage-sensitive Ca^{2+} channels in the acidosis-induced coronary constriction. Nelson *et al.* (1990) demonstrated that voltage-sensitive Ca^{2+} channels in vascular smooth muscle cells are very sensitive to alterations in membrane potential that occur in resistance arteries. In turn, the membrane potential of such cells is regulated by K^+ channels (Daut *et al.* 1994). Any manoeuvre which inhibits K^+ channels will depolarize the membrane, causing Ca^{2+} entry and contraction. Indeed, compounds which block a variety of K^+ channels have been demonstrated to cause contraction of

isolated porcine coronary arteries (O'Rourke 1996). In the present study it was shown that 4-AP, TBA, TEA Penitrem A and GLIB all increased CPP. This raises the possibility that acidosis could be blocking one or more K^+ channels in the coronary circulation of the rat and this would lead to an increase in CPP.

Acidosis has been demonstrated to inhibit a variety of K^+ channels in a variety of preparations. Acidosis (pH 6.8) had no effect on the channel conductivity of Ca^{2+}-activated K^+ channels in rat pancreatic β cells(Cook *et al.,* 1984). However, acidosis shifted the activation curves for the channels to the right. A similar inhibition of Ca^{2+}-activated K^+ channels by extracellular pH has been described in type I cells of the rat carotid body (Peers 1990). Intracellular acidosis produced by the addition of acetate also reduced current amplitude (Peers & Green 1991). Rat ventricular myocytes have also recently been demonstrated to contain a proton-sensitive transient outward K^+ current (Xu & Rozanski 1997).

Ahn & Hume (1997) describe the pH regulation of voltage-dependent K^+ channels in pulmonary artery smooth muscle. Interestingly, they describe differential effects of acidosis on whether it was intra- or extracellular in nature. Extracellular acidosis (pH 7.0-6.4) reduced current, produced a positive shift in the steady-state activation and reduced the maximum conductance in voltage-dependent K^+ channels. In contrast, butyrate-induced intracellular acidosis caused the opposite effects.

There have been reports of a proton titratable site controlling the conductance of K^+ channels in squid giant axon (Carbone *et al.,* 1978). Similarly, Sabirov *et al.,* (1997) using expressed inward rectifier channels described proton sensitive sites located on

both the inside and outside of the channel. This study demonstrated that the suppressive effect of protons was voltage-sensitive, suggesting that the proton-sensitive site was located closely to the outer entrance to the pore. The intracellular site, however, was voltage-independent. The same study also showed that extracellular protons reduced single channel conductance while intracellular protons decreased the effective number of available channels.

The location of the proton binding site which is responsible for alterations in conductivity and other channel characteristics is an enormous field of research. Most voltage-sensitive K^+ channels are tetrameric in structure with sub-units containing six putative transmembrane spanning segments. There is a highly conserved region between the S5 and S6 subunits which, in experiments performed with *Shaker*-type K^+ channels, is implicated in ion conduction. Replacement of isoleucine-369 with histidine infers a high sensitivity to blockade by H^+, with a pK_a of 6.4 (Kukulajan *et al.* 1995).

If this effect of acidosis on K^+ channels occurred in vascular smooth muscle, it would decrease the transmembrane K^+ current, depolarize the membrane leading to Ca^{2+} entry and thus contraction of the vascular smooth muscle. However, species difference need to be accounted for . Also, acidosis has been shown to block L-type Ca^{2+} channels in vascular smooth muscle which would physiologically oppose any effects of K^+ channel blockade.

This acidosis-induced reduction in K^+ current which could possibly lead to depolarization would have to overcome the acidosis-induced reduction in Ca^{2+} current. However, acidosis has been demonstrated to have only a modest effect on L-type Ca^{2+}

channels. In isolated porcine coronary artery smooth muscle cells, acidosis has been shown to have an inhibitory effect on Ca^{2+} channel current. Increasing the percentage CO_2 from 0 to 5% (25mM HCO_3^-) decreased the current by 34% compared to that measured in HEPES-buffered Tyrode (Klöckner & Isenberg 1994a). This study also proposed that the single channel conductance and the life time of channel openings were not influenced by intracellular pH.

A similar degree of inhibition of the current flowing through L-type Ca^{2+} channels has been observed in further study. In a separate study using isolated vascular smooth muscle cells from the basilar artery of the guinea pig, West *et al.* (1992) described a sigmoidal relationship between peak Ca^{2+} current and extracellular pH between the limits of 8.6 to 5.4. Acidosis (pH 6.8) only reduced the current by 30%. The basis of the proton-induced blockade of L-type Ca^{2+} channels has recently been investigated (Chen *et al.,* 1996a)

Therefore, acidosis has only a modest inhibitory effect on Ca^{2+} current flowing through L-type Ca^{2+} channels. The remaining 60-70% still constitutes a huge Ca^{2+} current. This may still be large enough to allow sufficient Ca^{2+} to enter the vascular smooth muscle cell and cause constriction. Therefore, acidosis has potentially two opposing effects: a possible depolarizing effect on membrane potential and an inhibitory effect on Ca^{2+} influx through L-type Ca^{2+} channels. If this modest inhibitory effect on the L-type Ca^{2+} could be overcome through membrane depolarization, then the acidosis-induced coronary vasoconstriction observed in the rat heart could be due to the acidosis-induced membrane depolarization.

4.12 *Relevance Of Single Cell Studies*

Reports of the relationships between pH and contractile force in vascular preparations are complex and often contradictory. Any comparisons between studies must be carefully drawn since experimental conditions can affect the results. The discrepancies between this and other studies could possibly be ascribed to experimental conditions.

As demonstrate in this study, contractile agents employed in studies can also have important implications in the observations. Rat isolated mesenteric arteries were unresponsive to lactate-induced dilations when pre-contracted with high K^+ solutions compared to noradrenaline-contracted preparations (McKinnon *et al.,* 1996). A similar preparation contracted by noradrenaline could be relaxed by acetylcholine via NO and membrane hyperpolarization. In contrast, preparations contracted by the thromboxane mimetic, U46619, could only be relaxed by NO (Plane & Garland 1996).

It must be borne in mind that these studies all utilized vessels of a certain diameter, in isolation. Recently, it has been demonstrated that ionic channels have a different distribution depending on the vessel diameter, and so may alter the response to certain stimuli (Archer *et al.,* 1996; Quayle *et al.,* 1996). Thus, it could be argued that the results from studies which use such preparations may not be representative of the entire vascular preparation.

Indeed, Halliday *et al.,* (1995) reported that studies on ionic currents in isolated vascular smooth muscle cells cannot easily predict the actions of the drug on intact preparations. Using rabbit aortic strip preparations and isolated vascular smooth muscle cells from the same vessel, it was demonstrated that no correlation existed between the effects of K^+ channel blockers on single cells ionic currents and tension recordings from intact preparations.

Single cells, and cell cultures, are valuable experimental tools since they are readily available, homogenous and reproducible. Intact, vascular preparations, however, have the advantage that the cell-to-cell communications are still present, with no enzymatic and minimum mechanical disturbance.

5.1 Future Work

5.1.1 Isolated perfused rat hearts

This study primarily investigated the effects of acidosis (both extra- and intracellular) on the tone of *in situ* coronary vessels in an isolated perfused rat heart. Further experiments could be performed to further characterise the effects of more vasodilatory agents, which act via different mechanisms of action, on the acidosis-induced constriction. In addition, in attempting to further investigate the possible role of K^+ channels in acidosis-induced coronary constrictions, different K^+ channel blockers could be applied in the presence of acidosis to see if any further constriction can be observed, as was seen with endothelin-1.

A similar approach could also be used to examine the possible differences between rat and guinea pig coronary circulations. The guinea pig coronary circulation only dilated in response to acidosis, whereas in the rat a transient dilation was followed by a sustained constriction. This probably reflects a fundamental difference between the tissues with respect to their response to acidosis *e.g.* lack of a pH-sensitive K^+ channel or vasoactive substance. It would be of great interest to investigate this difference in response in other species, in particular humans. Since acidosis is known to occur during myocardial ischaemia, and considering the prevalence of the condition in the Western world, any effect of acidosis on the coronary vessels could have a serious effect on the outcome of the condition.

5.1.2 Single Cell Studies

Of some interest are the transient increases in cardiac contractility observed only with application of metabolic acidosis. It would be of great interest to use isolated rat cardiac myocytes loaded with a Ca^{2+}- and pH-sensitive fluorescent indicators to investigate whether metabolic acidosis does cause a transient alkalinization similar to that already reported in rabbit cardiac myocytes (Nakanishi *et al.,* 1990) as alkalinization can increase contractility by sensitising the contractile proteins.

Results obtained with Ca^{2+} channel antagonists suggest that extracellular acidosis stimulated Ca^{2+} influx through voltage-sensitive channels leading to constriction. The successful isolation of single vascular smooth muscle cells from whole hearts would allow further investigations to be performed. In particular, it would be of interest to use pH-sensitive intracellular dyes and fluorimetric measuring techniques to investigate to what degree the experimental manoeuvres utilised to produce extracellular acidosis, plus butyric acid and NH_4Cl, affected intracellular pH and Ca^{2+}.

Similar cell isolation techniques could be useful to address the effects of the experimental procedures on ionic currents as measured by electrophysiological techniques. By measuring membrane potentials from coronary smooth muscle cells, the question of whether acidosis depolarizes the cell to stimulate L-type Ca^{2+} channels could be answered. In addition, by measuring specific ionic currents, whole cell K^+ and Ca^{2+}, one could ascertain the effects of acidosis on these crucial factors in smooth muscle contraction.

5.1.3 Rat Superior Mesenteric Bed

In response to the two constrictors used in the study, MPP increased to a similar level. However, this does not necessarily represent the relationship between cytosolic Ca^{2+} and tension. It would be of great interest to investigate this relationship with respect to the agents used here. The relative contribution of IP_3-induced Ca^{2+} release and direct Ca^{2+} influx leading to calmodulin-induced myosin phosphorylation, plus a possible sensitisation effect of phenylephrine could be compared to that of K^+-induced depolarization. Any difference in the relationship between these could underlie the differences observed for the same experimental manouevre,

6.0 ***References***

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